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Teresa Rita Pitorra Marta

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Dissertação para obtenção do grau de Mestre em
Engenharia Farmacêutica

Orientador: Professor Doutor José Cardoso Menezes
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Abstract

Perhaps no greater challenge exists for public health, patient safety, and shared global health security, than fake, falsified, fraudulent or poor quality unregulated medicines - also commonly known as “counterfeit medicines” - now endemic in the global drug supply chain (Tim K Mackey & Liang, 2013).

Counterfeit medicines pose a serious risk to public health around the world. It is low- and middle-income countries and those in areas of conflict, or civil unrest, with very weak or non-existent health systems that bear the greatest burden of SSFFC (Substandard, Spurious, Falsely labelled, Falsified and Counterfeit) medical products because the cost of legitimate drugs is beyond the reach of much of the population and legal controls are often weak (World Health Organization, 2010)**.** For this purpose, further scientific research and development is called for to design user-friendly, low-cost and robust portable (hand-held) devices and techniques for detecting and identifying counterfeit medicines in real settings (Karunamoorthi, 2014). This dissertation promotes a technique called Raman spectroscopy that has the potential to optimize quality testing for a broad range of medicines.

The aim of this dissertation was to study de detection of medicines through its package, for which it was relevant to investigate if there were differences within the package material and how these differences could affect the spectrums of the tablets under analysis. The results revealed that in fact there are variations in package materials that influence the spectrums acquired. These variations can be due to thickness or density variations. Nevertheless, these variations can be easily overcome using preprocessing methods. For this study, calibration tablets of paracetamol, were also made on purpose for experiencing the detection of different concentrations of an API through blister packages. Furthermore, in this investigation, three different probes were used for the measurements: PhAT probe, MKII probe attached to Raman Workstation microscope, and a green laser, to compare results. Results show that it is possible to detect different concentrations of an active ingredient of tablets through a white blister package using Raman spectroscopy, namely the PhAT probe. This equipment can be put in place analysis enabling faster detection of counterfeit medicines, in a non-invasive and non-destructive way that requires no sample preparation and does not need highly specialized personnel for its use. It provides reliable and useful data and it is portable. This is a promising technique for detecting counterfeit medicines and it is relatively low cost.

Key words: Counterfeit medicines; paracetamol tablets; blister packaging; PhAT probe; MKII probe; Green laser.

Resumo

Talvez não exista maior desafio para a saúde pública global do que a existência de medicamentos não regulamentados, de fraca qualidade, falsificados ou fraudulentos – também conhecidos como "medicamentos falsificados" - agora endémicos na cadeia global de abastecimento e distribuição de medicamentos (Tim K Mackey & Liang, 2013). A qualidade dos medicamentos disponíveis varia muito entre diferentes países devido à falta de regulamentos bem definidos e existência de práticas de controlo de qualidade deficientes. Os medicamentos falsificados incluem produtos com as substâncias adequadas ou com ingredientes errados, sem substância ativa, com quantidade insuficientes ou excessiva de substância ativa, ou com rotulagem errada e falsa. Por vezes, os medicamentos podem ser contaminados com outras substâncias ou podem até sofrer degradação química devido a fracas condições de armazenamento por exemplo em ambientes húmidos. Os medicamentos falsificados são amplamente distribuídos e podem ser bastante sofisticados, incluindo embalagens e estratégias de marketing muito convincentes.

Os medicamentos falsificados representam um sério risco para a saúde pública em todo o mundo, sendo que a maioria das denúncias estão relacionadas com antibióticos, anti protozoários, hormonas e esteroides. São os países em desenvolvimento e aqueles em áreas de conflito, ou agitação civil, com sistemas de saúde muito fracos ou inexistentes que mais sofrem com produtos médicos “SSFFC” (Substandard, Spurious, Falsely labelled, Falsified and Counterfeit - denominação atribuída pela Organização Mundial de Saúde, para a definição de medicamentos falsificados), uma vez que o custo dos medicamentos legítimos está fora do alcance de grande parte da população e o controlo legal dos medicamentos é muitas vezes fraco (World Health Organization, 2010). Os medicamentos falsificados nestes países aumentaram devido à existência de muitas doenças infecciosas como a malária e a tuberculose.

Neste estudo, é dado o exemplo do sistema de vigilância e monitorização da Organização Mundial de Saúde, que utiliza um sistema de inspeção constituído por três níveis: um primeiro nível realizado no local, que inclui uma inspeção visual da embalagem do medicamento em análise e a sua comparação com embalagens de medicamentos originais; um segundo nível, também este realizado no local, que se segue quando existem dúvidas relativamente aos testes realizados no primeiro nível e que inclui uma validação laboratorial utilizando métodos relativamente simples; se ainda assim os resultados forem inconclusivos segue-se o terceiro nível onde o medicamento é enviado para um laboratório forense, fora deste local, onde são realizados testes mais específicos de confirmação. Trata-se de um processe demorado pelo que mais investigação e desenvolvimento científico são requeridos para projetar técnicas de deteção e equipamentos fáceis de utilizar, de baixo custo e que sejam robustos e portáteis permitindo a identificação de medicamentos falsificados rapidamente e no local (Karunamoorthi, 2014).

Esta dissertação promove uma técnica chamada espectroscopia Raman, que tem o potencial para otimizar testes de qualidade para uma ampla gama de medicamentos.

Quando um feixe de luz interage com matéria, este pode ser transmitido, absorvido ou espalhado. Quando a luz é espalhada a partir de uma molécula, a maioria dos fotões é espalhada elasticamente, sendo que os fotões espalhados têm a mesma energia, frequência e comprimento de onda que os fotões incidentes. Contudo, uma pequena quantidade de luz é espalhada inelasticamente ou seja, a frequências diferentes, e normalmente mais baixas, do que os fotões incidentes. Este é chamado efeito Raman e foi descoberto por Krishna e Raman. Um espectro Raman contém bandas que são características e proporcionais a concentrações específicas de moléculas numa amostra, pelo que a espectroscopia Raman fornece uma boa análise qualitativa e quantitativa. A intensidade do espalhamento Raman é proporcional ao número de moléculas que produzem este espalhamento Raman. Como resultado, a intensidade do espalhamento Raman pode ser utilizada para medir quanto de um material está presente na amostra em análise (análise quantitativa). A forma de um espectro Raman pode ser utilizada para determinar que tipos de vibrações moleculares existem na amostra em análise. Esta informação vibracional pode ser utilizada para identificar materiais numa amostra (análise qualitativa). Diferenças em termos de stress, temperatura, estrutura cristalina, micro-heterogeneidade etc, podem, portanto, frequentemente ser medidas usando a espectroscopia Raman. A espectroscopia Raman é benéfica para diversos tipos de análise quantitativa e qualitativa num vasto número de campos, incluindo investigação química fundamental, ciências da vida (por exemplo, estudos biomédicos in vivo), controlo de processos, ciências forenses e na área farmacêutica. A espectroscopia Raman pode, deste modo, ser utilizada como uma tecnologia não destrutiva, não invasiva e ainda, como uma tecnologia de monitorização à distância. De um modo breve, as vantagens conhecidas da espectroscopia Raman incluem: elevada especificidade química, a capacidade de quantificar múltiplos constituintes numa forma farmacêutica sólida, a capacidade de analisar diferentes polimorfos e formas cristalinas; a elevada velocidade de análise; a ausência de necessidade de preparação da amostra; a ausência de necessidade de utilização de solventes e/ou consumíveis e a natureza de análise não destrutiva em comparação com outras técnicas de análise tradicionais. Portanto, a espectroscopia Raman é uma tecnologia estabelecida para assegurar a qualidade de produtos farmacêuticos permitindo identificar substâncias ativas e dar informação adicional sobre os excipientes, assim como a concentração relativa das substâncias ativas para os excipientes. Estes rácios podem ser a chave para detetar medicamentos falsificados uma vez que os indivíduos que produzem este tipo de produtos frequentemente têm em conta a quantidade da substância ativa mas não são tão precisos com as quantidades exatas de excipientes. Os instrumentos baseados na tecnologia do efeito Raman têm vindo a evoluir ao longo dos anos, passando de espectrómetros tradicionais de laboratório para ferramentas de menores dimensões, mais acessíveis em termos de custo, mais rápidas e eficientes, pelo que a análise de amostras em situações reais (em campo) ou até dentro de embalagens ou contentores, passou de um conceito ideal para uma tecnologia bem estabelecida para diferentes produtos farmacêuticos.

O objetivo desta dissertação é explorar a deteção de medicamentos falsificados utilizando técnicas de espectroscopia Raman para uma análise não destrutiva e não invasiva, tendo em

vista a sua aplicabilidade em campo (ou seja, em locais como fronteiras ou locais onde se realiza a inspeção e distribuição de medicamentos) e não, em laboratórios com boas condições e equipamentos assim como pessoal especializado. Esta investigação procura estudar a deteção de medicamentos falsificados através da sua embalagem, para a qual foi relevante investigar possíveis diferenças no próprio material de embalagem e o quanto essas diferenças poderiam interferir com os espectros obtidos dos comprimidos em análise.

Os resultados revelaram que, de facto, existem variações em materiais de embalagem que influenciam os espectros adquiridos. Estas variações podem dever-se a diferenças de espessura ou densidade no material. Ainda assim, concluiu-se que estas variações podem ser facilmente ultrapassadas através da utilização de métodos de pré-processamento dos espectros. Para este estudo, foram também produzidos comprimidos de paracetamol, no sentido de experimentar a deteção de diferentes concentrações de um princípio ativo, através da sua embalagem. Além disto, nesta dissertação, foram ainda utilizadas três sondas para as mesmas medições: a sonda PhAT, a sonda MKII conectada ao microscópio Raman Workstation, e um laser verde, para comparar os resultados. Os resultados mostram que é possível detetar concentrações diferentes de um princípio ativo em comprimidos, através de um blister branco, utilizando espectroscopia de Raman, mais precisamente, a sonda PhAT. Este equipamento pode ser colocado em qualquer local de análise, permitindo uma deteção rápida dos medicamentos falsificados, de modo não-invasivo e não-destrutivo sem necessitar de preparação prévia da amostra nem de pessoal altamente especializado para a sua utilização. Este equipamento fornece dados confiáveis e é portátil. Esta é uma técnica promissora para a deteção de medicamentos falsificados e apresenta um custo relativamente baixo.

Palavras-chave: Medicamentos falsificados; Comprimidos de paracetamol; Blister; sonda PhAT; sonda MKII; Laser verde.

Acronyms

AIDS	Acquired Immune Deficiency Syncrome
API	Active Pharmaceutical Ingredient
CCD	Charge-Coupled Devices
CD3	Cluster of Differentiation 3
DART	Direct Analysis in Real Time
DESI	Desorption Electrospray Ionazation
ERP	Enterprise Resourse Planning
FTIR	Fourier Transformed Infrared
FT-Raman	Fourier Transform-Raman
GMP	Good Manufacturing Practices
GPHF	German Pharma Health Fund
HCA	Hierarchical Cluster Analysis
HIV	Human Immunodeficiency Virus
HPLC	High-performance Liquid Chromatography
HPTLC	High-performance Thin Layer Chromatography
IMPACT	International Medical Products Anti-Counterfeiting Task Force
IR	Infrared
IT	Information Technology
MCR-ALS	Multivariate Curve Resolution – Alternating Least Squares
MS	Mass Spectrometry
Nd:YAG	Neodymium-Yttrium Aluminium Garnet
Nd:YVO4	Neodymium-Yttrium Ortho-Varradate
NIR	Near Infrared
NMR	Nuclear Magnetic Resoance

NMRA	National Medicines Regulatory Authorities
OOS	Out Of Specification
PAT	Process Analytical Technology
PC	Principal Component
PCA	Principal Component Analysis
pH	Potential of Hydrogen
PhAT	Pharmaceutical Area Testing
PLS	Partial Least Squares
RFID	Radio Frequency Identification
RRS	Resonance Raman Spectroscopy
SERS	Surface Enhanced Raman Spectroscopy
SERRS	Surface Enhanced Resonance Raman Spectroscopy
SG	Savitzky-Golay
SNR	Signal to Noise Ratio
SNV	Standard Normal Variate
SORS	Spatially Offset Raman Spectroscopy
SSFFC	Substandard, Spurious, Falsely labelled, Falsified and Counterfeit
TLC	Thin-Layer Chromatography
TRS	Transmission Raman Spectroscopy
UV	Ultraviolet
WAI	Wide Area Illumination
WHO	World Health Organization
XRD	X-ray Diffraction

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Chapter 1 - Introduction

Perhaps no greater challenge exists for public health, patient safety, and shared global health security, than fake, falsified, fraudulent or poor quality unregulated medicines - also commonly known as “counterfeit medicines” - now endemic in the global drug supply chain (Tim K Mackey & Liang, 2013). The World Health Organization (WHO) defines counterfeit products as medicines that are deliberately and fraudulently mislabeled with respect to identity and/or source. Counterfeit medicines include products with correct ingredients or with wrong ingredients, without active ingredients, with insufficient or excessive amount of the active ingredient, or with false or misleading labeling.
There are also “substandard medicines” (also called out of specification (OOS) products) which are genuine medicines produced by manufacturers authorized by the National Medicines Regulatory Authorities (NMRA) which do not meet quality specifications set for them by national standards (WHO, 2009). They arise mostly due to the application of poor manufacturing practices by the producer or when a good quality medicine is stored and distributed under improper conditions leading to deterioration of the quality of the product (WHO, 2009).

Both substandard and counterfeit medicines are serious problems, and remain as one of the most neglected public health issues, where counterfeiting is mounting to more than 60% in third world countries (Karunamoorthi, 2014). Counterfeit medicines pose a serious risk to public health around the world. The issue of counterfeits has no single or simple solution and cannot be eliminated by any one individual, organization or government. It is a global problem that needs a global, collaborative approach (Shore & Pfizer, 2015). Currently a number of different approaches are being undertaken to identify counterfeit medicines. Since technology may improve the detection of low-quality drugs, it would be important that these methods will also be available in developing countries, where counterfeit products are the most common (Kelesidis & Falagas, 2015). For this purpose, further scientific research and development is called for to design user-friendly, low-cost and robust portable (hand-held) devices and techniques for detecting and identifying counterfeit medicines in real settings (Karunamoorthi, 2014).
This dissertation promotes a technique called Raman spectroscopy that has the potential to optimize quality testing for a broad range of medicines.

When light interacts with matter, it can be transmitted, absorbed or scattered. When light is scattered from a molecule, most photons are elastically scattered. The scattered photons therefore have the same energy, frequency and wavelength as the incident photons. However, a small fraction of light is inelastically scattered i.e. at frequencies different from, and usually lower than, the frequency of the incident photons. This is called Raman effect and it was discovered by Krishna and Raman. Raman scattering can occur due to a change in vibrational, rotational or electronic energy of a molecule (Kaiser Optical Systems, 2004). The spectrum of this wavelength-shifted light is called the Raman spectrum. The Raman spectrum contains bands that are characteristic and proportional to concentrations of specific molecules in the sample, thus Raman provides a good basis for qualitative and quantitative analysis.

The instruments used for Raman spectroscopy used to be large and complicated, and the experiments could be quite complex. However, in the past decades new developments in the Raman instrumentation (e.g. improved lasers, introduction of charge-coupled devices (CCD) detectors and fiber optics) have expanded the possibilities for the use of Raman spectroscopy. With these advances, fundamental problems of a weak Raman signal and fluorescence interference can be overcome (Lotte Boge Lyndgaard, 2013). Raman spectrometers have become smaller, sometimes even portable, and easy-to-use, with an analysis time reduced to seconds. Furthermore, non-destructive analysis can be provided without the necessity of sample preparation. The complex and vast amount of data generated using Raman spectroscopy often demands multivariate data analysis approaches (Paudel, Raijada, & Rantanen, 2015). With multivariate methods, it is possible to investigate the relations between all variables in a single context. These relationships can be displayed in plots as easy to understand as time traces and pair-wise scatter plots (Guide, 2013).

The aim of this dissertation is to explore the detection of counterfeit medicines using Raman spectroscopy techniques for non-invasive and non-destructive analysis, in view of its applicability *in the field* (hence, in locations such as borders or places where inspection and distribution of medicines is carried out) and not in laboratories with good resources as well as specialized personnel. This investigation seeks to study de detection of medicines through package, for which it is relevant to investigate if there are differences within the package material and how these differences affect the spectrums of the tablets under analysis. Aluminum blisters were also investigated to find some signal through this material. Calibration tablets of paracetamol, were made for experiencing the detection of different concentrations of an API through blister packages. The blisters used were white blisters of "*Ben-u-ron* tablets" from *Bene Farmacêutica*. Furthermore, in this investigation three different probes on two different instruments were used for the measurements: the PhAT probe and MKII probe attached to Raman Workstation microscope, and a green laser (cheaper but less accurate) to compare results.

This dissertation starts with an introduction to the illegal business of counterfeit medicines - **Chapter 2**. This chapter aims to briefly elucidate the readers of this dissertation regarding counterfeit medicines and their impact on society, as well as to describe some of the other techniques used for pharmaceutical analysis, in addition to spectroscopic methods.
Chapter 3 includes an introduction to the Raman effect, instrumentation, spectral interpretation and data analysis. It also includes a summary of studies regarding Raman spectroscopy for detecting counterfeit medicines. **Chapter 4** begins with a description of an investigation into differences within packaging material. After this, the production process of the paracetamol tablets was also described, followed by the description of the method of analysis of the tablets through the white blister pack with each probe. After the measurements, spectra were analyzed using *Latentix™ software*. *Latentix™* was developed with the intention to make low cost professional software for multivariate analysis so that Chemometrics and multivariate models

could be available to everybody in all technological and scientific disciplines, where complex data tables need exploration or efficient data analysis. This idea connects with the idea inherent to this dissertation which is the investigation of a method of analyzing medicines that is easy to use for people without a very high level of training. Finally, the conclusions of this investigation are presented in **Chapter 6**.

Chapter 2 - Counterfeit Medicines

Since the theme of this dissertation is using Raman spectroscopy methods for detecting counterfeit medicines, it is relevant to describe this widespread illegal business which affects millions of people worldwide, especially in developing countries. This chapter aims to briefly elucidate the readers of this dissertation regarding counterfeit medicines and their impact on society, as well as to describe some of the other techniques used for pharmaceutical analysis, in addition to spectroscopic methods.

2.1 What counterfeit/substandard medicines are

Perhaps no greater challenge exists for public health, patient safety, and shared global health security, than fake, falsified, fraudulent or poor quality unregulated medicines - also commonly known as “counterfeit medicines” - now endemic in the global drug supply chain. Counterfeit medicines are prevalent everywhere, from traditional healthcare settings to unregulated sectors, largely driven by the Internet. These dangerous medicines are expanding in both therapeutic and geographic scope, threatening patient lives, leading to antimicrobial resistance, and profiting criminal actors (Tim K Mackey & Liang, 2013).

The quality of commercially available drugs varies greatly among countries, due to the lack of strict regulations and to deficient quality control practices. In some countries, even the amount of active ingredient may be incorrect. In addition, poor formulation techniques can greatly affect the quality of medicine by slow-release of active ingredients (Karunamoorthi, 2014). Sometimes drugs may be contaminated with other substances, and via poor storage conditions, where humid, tropical environments may contribute to chemical degradation of several pharmaceuticals (Hall et al., 2006).
Counterfeit medicines are widely distributed and self-prescribed (incorrectly and correctly). They can be highly sophisticated, including convincing packaging, holograms and marketing, and represent a major technical and law enforcement challenge.

The WHO defines counterfeit products as medicines that are deliberately and fraudulently mislabeled with respect to identity and/or source. Counterfeit medicines include products with correct ingredients or with wrong ingredients, without active ingredients, with insufficient or excessive amount of the active ingredient, or with false or misleading labeling.

Unfortunately, efforts to improve the quality of medicines in developing countries are being hampered by confusion over the terms used to describe different types of poor quality medicines (Newton et al., 2011). The importance of a universal definition of a counterfeit medicine is of great importance since it is a problem that affects many countries and varies from country to country. A universal definition facilitates the comprehension of the true extent of the problem at a global level allowing the development of global strategies to combat the problem.

The WHO defines counterfeit medicines as: Substandard, Spurious, Falsely labelled, Falsified and Counterfeit (SSFFC) medical products (Organization, n.d.). Counterfeit medicines can be classified in four different subclasses:

• "**perfect**": these products contain the correct active ingredient and excipients in the right amount, but with the wrong name of manufacturer and/or country of manufacture indicated on the label. They are often manufactured in foreign countries and illicitly imported (parallel marketing), thus resulting in an economic damage.

• "**imperfect**": these products contain the right components, with an incorrect concentration and/or formulation resulting in defective quality specifications. In the vast majority of cases, they are devoid of any therapeutic efficacy.

• "**apparent**": they are similar to the original product, but contain non-active ingredients or foreign substances. Usually, apparent counterfeits are copies of medicinal products that are not the only ones used for the cure of a particular illness.

• "**criminal**": they are apparently similar to the original medicinal product, but do not contain any active ingredient and can even include harmful or toxic substances. They are usually sold at high prices and for treatment of serious pathologies. Consequences for users of criminal counterfeits can be fatal. (Baratta, Germano, & Brusa, 2012) (WHO, 2009)

WHO estimates about 60% of purchased counterfeited products did not have any active pharmaceutical ingredient (API), 17% contain too much or too little API, whilst another 16% contain the wrong ingredients altogether (Karunamoorthi, 2014).

Substandard medicines are genuine medicines produced by manufacturers authorized by the NMRA which do not meet quality specifications set for them by national standards (WHO, 2009). They arise mostly due to the application of poor manufacturing practices by the producer or when a good quality medicine is stored and distributed under improper conditions leading to deterioration of the quality of the product. A substandard medicine can be considered as counterfeit if a legitimate manufacturer gets involved in a criminal activity and produces a substandard product intentionally or deliberately (WHO, 2009). After all, what makes a product counterfeit is the criminal act involved. Both substandard and counterfeit medicines are serious problems, and remain as one of the most neglected public health issues, where counterfeiting is mounting to more than 60% in third world countries (Karunamoorthi, 2014).

Figure 2.1 An official unloads counterfeit medicines for destruction in Lagos, on October 13, 2001. At best, falsified prescription medicines have no effect, acting like placebos, but at their worst, they are highly toxic. Either way they bring in vast sums of money for those behind the illicit traffic ("Fox News World," 2013).

2.2 What encourages counterfeiting of medicines

WHO has determined factors that may lead to the spread of substandard drugs, such as counterfeiting, chemical instability, and poor quality control (Kelesidis & Falagas, 2015). Table 2.1 presents a summary of factors that contribute to the spread of low-quality medicines Corruption, crime, and poorly regulated pharmaceutical companies are major contributors to counterfeiting. According to the International Drug Industry Federation the counterfeit drug market is more profitable for counterfeiters than that of illicit drugs (Hall et al., 2006).

Medicines are attractive for counterfeiting, because they are high-value products in relation to their bulk and the demand for medicines is endless. It is possible to produce counterfeit drugs without the existence of special facilities. They can be produced in small industries, at home, in backyards or under the shade of a tree. For a counterfeiter, the cost of excipients may become financially accessible if replaced by low cost excipients, or if they are omitted altogether, as often happens. Counterfeiting of medicines is even more successful if the counterfeit medicine is copied to make it look like the original product. Patients and/or buyers are not able to detect whether the product they are buying is of good quality let alone to detect if the product is counterfeit (WHO, 2009).

The development, manufacture, importation and subsequent handling of medicines within the distribution channels should conform to prescribed standards, and the quality of medicines should be rigorously controlled. However, this would require strong NMRAs. This in turn needs strong government, will and commitment to provide adequate human, financial, and other resources, appropriate infrastructure and legal power to enforce medicine regulation. **In many WHO Member States the political will and commitment to establish strong NMRAs or to strengthen existing ones is weak or lacking**. Consequently, medicine regulation and control activities are ineffective and inefficient resulting in noncompliance of local manufacturing with requirements of good manufacturing practices, circulation of unregistered/unapproved medicines in the national market, smuggling of medicines through port of entries and boarders, etc (WHO, 2009).

Legislation and regulations are the basis for medicine regulation. In countries where **laws and regulations do not exist or are inadequate for proper control of medicines,** criminals are encouraged to produce counterfeit medicines. Currently, only some of WHO's Member States adopted special national legislation on counterfeit medicines. Moreover, the sanctions imposed on counterfeiters are weak and do not constitute an impediment to this illegal business. The absence of legislation prohibiting the manufacture and trade of counterfeit drugs or the absence of deterrent penalties encourages counterfeiters since there is no fear of being arrested and prosecuted. Currently, among the 193 WHO Member States about 20% are known to have well developed regulating drugs. Of the remaining Member States, about 50% implement drug regulation at different levels of development and operational capacity. The remaining 30% do not have any kind of drug regulation strategies or have poorly structured regulations that hardly work (WHO, 2009).

Regulatory and law enforcement systems remain weak. Enacting deterrent legislation alone will not solve the problem of counterfeit medicines. Legislation needs to be enforced. Where existing legislation is not enforced crime is perpetuated as criminals are not afraid of being arrested and prosecuted. Counterfeiters are opportunistic criminals motivated by profits yet with limited legal penalties. In most countries cooperation between NMRA, police and customs is either weak or non-existent, which makes it difficult to detect, arrest and bring criminals to trail (WHO, 2009).

Furthermore the detection of counterfeit medicines is difficult because medical personnel attribute the problem of drugs that produce poor clinical outcomes to patient variation (Delepierre, Gayot, & Carpentier, 2012). In most cases, patients are unlikely to suspect that they are using counterfeit drugs. Often, drug packaging is thrown away, which makes it difficult to test for bad drugs, particularly because the drugs may be undetectable in the bloodstream after a few days. Also, patients may not want to reveal that they have bought drugs without a prescription over the Internet. Therefore, the probability of a counterfeiter getting caught can be very low (Rudolf & Bernstein, 2004).

Efforts by a variety of public and private sector entities, national governments, and international organizations have made inroads in combating this illicit trade, but are stymied by ineffectual governance and divergent interests (Tim K Mackey & Liang, 2013).

The efficiency of personnel working in medicine regulation and those involved in law enforcement activities such as police, customs and the judiciary, is adversely affected by **conflict of interest and corruption** resulting in laws not being enforced and criminals not being arrested, prosecuted and convicted for their crimes. In order to address the problem corruption, governments need to develop policies on conflict of interest and establish mechanisms for managing conflict of interest. Personnel working in medicine regulation as well those participating in national medicines anti-counterfeiting programs should be required to sign conflict of interest form.

Empowering consumers and public interest groups to participate in medicine regulation, making NMRAs transparent and accountable and motivating NMRA staff and enforcement officers by providing them incentives can help in combating conflict of interest and corruption (WHO, 2009).

When the **supply of medicines in a country is short or erratic**, patients and consumers tend to look for alternative sources. Such situations encourage criminals to smuggle in medicines or manufacture counterfeit medicines and distribute them as a substitute for genuine medicines (WHO, 2009). Many of the drugs in short supply are lifesaving anticancer drugs, and this promotes the development of counterfeits that can result in considerable suffering or death for the patient. Drug shortages lead to increased prices for a legitimate drug and increase the opportunity and ability for unscrupulous people to make financial gains by introducing counterfeit drugs into the market (Cherici, Mcginnis, & Russell, 2011). Counterfeiters are able to take advantage of the drug shortage and charge exorbitant prices (Rudolf & Bernstein, 2004).

Consumers who use medicines inappropriately generate demand for such medicines, the sources of which may be counterfeit. For example, the misuse of creams containing steroids for skin bleaching, and body building medicines has generated a market for counterfeit steroid-containing medicines. Often, these medicines are distributed through unauthorized channels or illicit markets (WHO, 2009).

Authentic medicines are often expensive, and people buy inexpensive medicines from unauthorized retailers, which are often considered of good quality by the public (Basco, 2004).
When price differences exist between identical products, patients and consumers go for the cheaper ones. This creates a greater incentive for counterfeiters to supply cheap counterfeit medicines (WHO, 2009). Less-developed countries are often a target for counterfeiters, since the cost of legitimate drugs may be unaffordable for a large proportion of a sizable population, and consequently patients prefer to use cheaper versions than expensive, genuine ones (Karunamoorthi, 2014).

Inter-sectoral cooperation between NMRAs, police, and customs services and the judiciary is essential for effective control of the national medicine market and enforcement of medicine legislation. When such **cooperation between stakeholders is ineffective**, counterfeiters can escape detection, arrest and penal sanctions (WHO, 2009).

Detecting counterfeits is often difficult, because many of these goods pass through a long and complicated distribution network, thereby creating opportunities for counterfeits to enter the legitimate supply chain (Rudolf & Bernstein, 2004).
Despite clear global public health threats, surveillance for counterfeit medicines remains extremely limited, with available data pointing to an increasing global criminal trade that has yet to be addressed appropriately (Tim K Mackey & Liang, 2013). When **countries do not control**

export medicines to the same standard as those produced for domestic use, criminals find it easy to manufacture and export counterfeit medicines. Medicines are also traded in free-trade zones or free ports where control is lax or absent. In such places medicine products are sometimes re-labelled in order to hide their true source and identity (WHO, 2009).

Table 2.1 Summary of Factors That Contribute to the Spread of Low-Quality Medicines (Kelesidis & Falagas, 2015)

Substandard medicines	Counterfeit medicines
Reduced stability of medicines in developing countries due to environmental conditions and poor storage	Financial interests: crime, corruption of politicians and industry
No good manufacturing process in developing countries	Inadequate resources dedicated to control manufacture, import, and export activities, complex transactions, inefficient cooperation among stakeholders
Poor quality control during manufacture	High demand for antimicrobials and vaccines exceeds supply
Poor surveillance about expiration dates and storage conditions in poor settings	High prices of original drugs
Use of non-standardized pharmacopoeias by many developing countries	Development of Internet
-	High rate of illiteracy and very low income of population in less-developed countries
-	Lack of sensitization of people in less-developed countries to the impact and dangers of counterfeit drugs purchased from non-authorized dealers

2.3 Impact of the production and distribution of counterfeit medicines in society

One of the most complex and challenging problems faced as a result of the globalization of health-care delivery is securing the integrity and safety of the global medicines supply chain. Dangerous forms of pharmaceuticals are illicitly sold by criminal elements and illegal transnational organizations creating dangers for patient safety and public health that undermine public and private investments in health care (T. K. Mackey, Liang, York, & Kubic, 2015). Poverty, weak economies, poor regulatory systems, short supply, and the rising cost of therapeutic agents have created a corresponding increase in production of fake drugs because of the huge profit margin (Karunamoorthi, 2014). The scope of this illegal international trade is broad and complex, includes products spanning a host of therapeutic classes and lifesaving treatments, leading to consequences in all populations from the poorest to wealthiest (T. K. Mackey et al., 2015).

Medicines, including vaccines, save lives and prevent diseases and epidemics only if they are safe, efficacious, of good quality and are used rationally. According to reports received from

national authorities in countries and information published in newspapers and journals, both well established (generic) medicines and innovative medicine products are affected including very expensive products for cancer to very inexpensive products for treatment of pain. Generally, high volume (high consumption) and expensive medicines are the main targets of counterfeiters (WHO, 2009). The WHO estimated that nearly half of the global pharmaceutical market is occupied by counterfeit drugs (Karunamoorthi, 2014).

Counterfeit medicines involve both lifesaving and lifestyle drugs. In general, the industrialized economies suffer with life-style related diseases (for example erectile dysfunction or baldness), whereas in the emerging economies infectious diseases are a matter of grave concern (Karunamoorthi, 2014). The largest number of reports relate to antibiotics, antiprotozoals, hormones and steroids. In developing countries, antibiotics and aniprotozoals such as anti-malarial medicines are commonly counterfeited. In developed countries hormones and steroids account for the majority of the cases reported (WHO, 2009). SSFFC medical products are often produced in very poor and unhygienic conditions by unqualified personnel, and contain unknown impurities and are sometimes contaminated with bacteria. (Figure 2.2)

Figure 2.2 On the left a counterfeit version of Pfizer's Viagra manufactured in China was distributed over the Internet by a network that included hundreds of brokers in the US and EU. On the right an illegal factory in Colombia washes empty vials before reuse in the production of counterfeit Tazocin, also a Pfizer antibiotic (Shore & Pfizer, 2015) *(Barry, 2014)*

Counterfeit medicines have flourished due to the emergence and resurgence of many infectious diseases, particularly the three major killer diseases: HIV/AIDS, malaria and tuberculosis (Karunamoorthi, 2014). Substandard/counterfeit antimicrobial drugs (including antibiotics, antituberculosis drugs, antivirals, vaccines and antimalarials) represent an expanding problem throughout developing countries with considerable consequences for global public health (Kelesidis & Falagas, 2015). It has been estimated that fake drugs for malaria and tuberculosis potentially kill over 700,000 people every year (Karunamoorthi, 2014).

The most common type of substandard/counterfeit antimicrobial drugs have a reduced amount of the active ingredient, and the majority of them are manufactured in Southeast Asia and Africa. These counterfeit medicines may cause increased mortality and morbidity and pose a danger to

patients. Antimalarials, followed by antibiotics, are the most common targets for counterfeiting (Kelesidis & Falagas, 2015). Counterfeit and substandard **antibiotics** can cause treatment failure that leads to increased mortality in humans. An excessive dose of the active ingredient in low-quality antimicrobials may be toxic to humans, especially in children or with antimicrobials with a narrow therapeutic range (Kelesidis & Falagas, 2015). Counterfeit antimicrobials such as **antimalarials** may have dangerous components or infectious contaminants, which can cause side effects. For example, injectable counterfeit antibiotics may contain methanol, a potentially lethal product for humans which may be responsible for pancreatitis, blindness, coma, cardio-circulatory failure, and death (Delepierre et al., 2012). The counterfeiting of antimalarials represents a form of attack on global public health in which fake and substandard products effectively serve as weapons of mass destruction, particularly in resource-constrained endemic settings, where malaria causes nearly 660,000 preventable deaths and threatens millions of lives annually. This crime against humanity is often underestimated or ignored (Karunamoorthi, 2014).

With the exponential increase in internet connectivity those engaged in the manufacture, distribution and supply of SSFFC medical products have gained access to a global market place (WHO, 2009). The Internet has become an important and convenient means for consumers to shop and save money. However, websites that traffic counterfeit goods have flourished on the Internet, creating confusion between which sites sell authentic goods and which do not (Rudolf & Bernstein, 2004). A culture of self-diagnosis and self-prescribing has led to the emergence of thousands of unregulated websites providing unsupervised access to SSFFC medical products. Purchase of medicines via the Internet has a high chance of exposing patients/consumers to counterfeit and substandard medicines. The patient/consumer has no way of knowing if the product is manufactured by an authorized manufacturer; if manufacturer complies with Good Manufacturing Practices (GMP) requirements or the products are approved by the NMRA of the country claimed to be country of manufacture (WHO, 2009). In more than 50% of cases, medicines purchased over the Internet from illegal sites that conceal their physical address have been found to be counterfeit, according to WHO (World Health Organization, 2010). Moreover, some of these Internet sites are linked to terrorist groups, such as Hezbollah and Al Qaeda, and others are linked to organized crime - posing a threat to national and international security (Liang, 2006). In an attempt by these sites to make even more profit, consumers may face other consequences of an online drug purchase, including credit card fraud, identity theft, and computer viruses (“U.S Food & Drug Administration,” 2013).

The true extent of the problem of counterfeit medicines is not really known, due to the fact that not every case is reported by competent authorities or manufacturers. They can be found in illegal street markets, via unregulated websites through to pharmacies, clinics and hospitals (Organization, n.d.). The international trade presents easy opportunities for counterfeiters to insert their products into the supply chain of legitimate pharmaceuticals and to disguise the source. Even in hospitals, there have been seen deliveries where counterfeit medicines have been added

to genuine batches of medicines. The counterfeiters simply falsify the delivery papers by adding an extra zero to the quantity supplied, then they make up the difference by adding their own boxes to the order (World Health Organization, 2010).
One case that illustrates this problem is that of a patient who was treated with injections for anemia, after a liver transplant. After 8 weeks of injections, the patient was still not responding to treatment. The treating physicians discovered that the medicine the patient used was counterfeit. In such cases, the consequences of counterfeits can be serious (Rudolf & Bernstein, 2004). The implications for patients are clear: counterfeits can kill.

Counterfeit medicines are widespread in all countries around the globe and represent a major public health concern, often resulting in treatment failure, serious deterioration of the state of health, or even death (Baratta et al., 2012). What was once considered a problem suffered by developing and low income countries has now become an issue for all. Globalization of the market in APIs and finished medical products brings with it the urgent need for effective International collaboration, cooperation and coordination to ensure global access to safe, quality, efficacious and affordable medical products. Several international and national strategies are required to efficiently detect counterfeits and combat this very important public health issue.

Counterfeit medicines are spread all over the world however, it is low- and middle-income countries and those in areas of conflict, or civil unrest, with very weak or non-existent health systems that bear the greatest burden of SSFFC medical products (Baratta et al., 2012). Developing countries are an obvious target for counterfeiters, because the cost of legitimate drugs may be beyond the reach of much of the population and legal controls are often weak, analysts say (World Health Organization, 2010). Even though higher-income countries have stringent regulations and better law enforcement, they also offer great rewards. According to the Medicines and Health care products Regulatory Agency in the United Kingdom, counterfeiters now also target the most lucrative markets, copying high-value, high-turnover, high-demand drugs (World Health Organization, 2010).

The use of substandard products may lead to under-dosing of antibiotics, which can increase **antimicrobial resistance**. In the case of the counterfeit drug that has no active ingredient, the drug fails to help the patient get better, which can ultimately harm the patient. In the case of antibiotics, for example, this can promote antibiotic resistance and the use of stronger antibiotics because physicians would believe that the first-line drug was not working, not knowing that the patient had been taking a counterfeit drug (Liang, 2006). As a result, in some developing countries multidrug-resistant bacteria may emerge, and the development of travel may further promote the spread of drug-resistant bacteria worldwide. Furthermore, therapeutic failure prolongs the period of contagiousness and increases the prevalence of infections from multidrug-resistant pathogens in the community. Antimicrobial resistance may also lead to increased morbidity that may further adversely affect the economies of governments. The financial burden on consumers who have to

pay for more expensive drugs, and on companies producing the original products, can be significant (Kelesidis & Falagas, 2015).

Low-quality antimicrobials may significantly decrease confidence in the efficacy of certain antibiotics. Poor-quality antimicrobials may lead physicians to lose confidence in specific antibiotics and thus to use broad-spectrum antibiotics as the drugs of choice for infections. According to the WHO, this may lead to loss of efficacy of relatively inexpensive drugs and will promote the use of more expensive antibiotics that patients in developing countries are not able to afford (Kelesidis & Falagas, 2015).

Intellectual property represents original, creative works and innovations who belonging to inventors, artists, musicians, and authors who, and businesses that, create something or acquire rights to a creation. The exclusive rights and legal protections of intellectual property come in the form of copyrights, trademarks, and patents. Innovation is important to economic growth and competitiveness in the global marketplace. Intellectual property protections provide the ability for society to prosper from innovation. These protections encourage innovation and creativity, but the rewards for innovation and creativity can be undermined by widespread theft associated with counterfeiting and trafficking of pirated goods (Rudolf & Bernstein, 2004). Although pharmaceutical companies spend considerable amounts of money on the research and development (R&D) of new drugs that benefit society and are approved as safe and effective, suppliers of counterfeit drugs bypass this process and supply these drugs at little cost to them; profit margins are often as high as 3000% (Kerry Capell, Suzanne Timmons in Bogotá, Jonathan Wheatley, 2001).

The threat of counterfeit pharmaceuticals is not new; many national authorities waged a long time their own fight against this illegal business. WHO has been working actively on this complex and politically sensitive issue since it was first discussed in May 1998 at the World Health Assembly. The efforts were intensified in 2006 when it launched the International Medical Products Anti-Counterfeiting Task Force (IMPACT), drawing members from international organizations, enforcement agencies, industry and non-governmental organizations (World Health Organization, 2010). WHO works with Interpol to dislodge the criminal networks raking in billions of dollars from this cynical trade (World Health Organization, 2010). Results from a string of law enforcement operations around the world by WHO and International Criminal Police Organization (Interpol) are slowly building a profile of the trade that shocks even regulators familiar with the issue. Health experts believe such operations have only scratched the surface of a flourishing industry in counterfeit medicines that poses a growing threat to public health around the world (World Health Organization, 2010). There have been found counterfeit versions of antibiotics, birth-control medicines, organ transplant medicines, medicines for the treatment of heart diseases, schizophrenia, diabetes and cancer.

2.4 Combating the business of counterfeit medicines

Counterfeit medicines pose a serious risk to public health around the world. The issue of counterfeits has no single or simple solution and cannot be eliminated by any one individual, organization or government. It is a global problem that needs a global, collaborative approach (Shore & Pfizer, 2015). Counterfeit medicines should be defined in terms of harm to health, with punishments appropriate for the injury or killing of patients. It is imperative that public health institutions, ministries, and lawyers, take the strategic lead in countering poor quality medicines (Newton et al., 2011). Governments must regulate the manufacture, export, import, storage, distribution, supply and sale of medicines to ensure the safety, efficacy and quality of medicines (WHO, 2009).

In order to combat the problem in an international level there should timely exchange of information on counterfeit medicines between, medicine regulatory authorities, pharmaceutical manufacturers, national law enforcement officers, international organizations such as WHO, Interpol and World Custom Organization; there should be more cooperation between all interested parties to develop harmonized measures to prevent the spread of counterfeit medicines globally. Cooperation would improve if all countries adopt a common definition of counterfeit medicines (WHO, 2009).

There are several safety precautions that should be taken in all countries to prevent the spread of counterfeit medicines. Table 2.2 shows examples of important and relatively simple actions that can be implemented, which hinder the illegal business of counterfeit medicines.

Table 2.2 Suggestions on How to Reduce Medicines Counterfeiting (adapted from (Rudolf & Bernstein, 2004) and (WHO, 2009))

• Increase public awareness, especially concerning Internet pharmacies
• Construct Internet search algorithms so that legitimate online pharmacies appear first
• Encourage more voluntary cooperation from companies along the Internet chain, such as credit card companies, Internet service providers, and couriers
• Improve the availability and affordability of medicines
• Improve management of supply chain
• Apply stiffer fines and jail sentences for convicted sellers of counterfeit medicines
• Increase due diligence by physicians when purchasing drugs and stiffer penalties for those who knowingly provide counterfeit medicines to their patients
• Inspect the informal market to prevent any illegal trade in medicines
• Improve cooperation with foreign governments regarding counterfeiting medicines
• Improve use of technology to track and trace counterfeiting medicines

Consumer Education Needed

Consumers should be encouraged to know where the online pharmacy is actually located, and the actual source of their drug supply. In addition to verifying the location of an online pharmacy, consumers should also mistrust of any company that will distribute medicines without a prescription. Consumers should also be informed about the extent of counterfeit drugs and the harm they cause. Finally, consumers should be encouraged not to buy prescription medicines without a valid prescription (Rudolf & Bernstein, 2004). It is also important to encourage consumers to report to their prescribers or physicians any lack of improvement in their health status in spite of the treatment or any adverse reactions experienced (WHO, 2009).

Track and trace counterfeiting medicines

Currently a number of different approaches are being undertaken to identify counterfeit medicines. Since technology may improve the detection of low-quality drugs, it would be important that these methods will also be available in developing countries, where counterfeit products are the most common (Kelesidis & Falagas, 2015). Some countries have already implemented track and trace and authentication technologies throughout their supply chains or at the point of dispensing the medicines. Field screening technologies have been implemented in a number of countries, with regulatory and Customs personnel trained to use hand held equipment in the assessment of the authenticity and quality of medicines ("World Health Organization," n.d.-a). Lack of inexpensive tools is one of the major impediments for their elimination/eradication. Further scientific research and development is called for to design user-friendly, low-cost and robust portable (hand-held) devices and techniques for detecting and identifying counterfeit medicines in real settings (Karunamoorthi, 2014).

WHO Surveillance and Monitoring System

WHO designed a global reporting system. The system is designed for use by trained focal points in National Medicine Regulatory Authorities. Reports of SSFFC medical products are submitted to the WHO via an electronic rapid alert form, currently available in English, French, Spanish and Portuguese languages. Some Member States submit reports of suspected SSFFC medical products and others submit reports of validated SSFFC medical products. When a report is received at WHO it is automatically uploaded in to a secure WHO database, and immediately compared against all other reports. Any matches are identified and details shared with the reporting Member State. WHO will contact the reporting focal point within 72 hours for further details and where requested provide technical support. In emergencies this may take the form of facilitating urgent laboratory analysis or in extreme and complex cases deploying experts in the field ("World Health Organization," n.d.-b).

A three-level approach consisting of different quality control procedures has been proposed to detect substandard/ counterfeit medicines (Figure 2.3) (Kelesidis & Falagas, 2015). **Level 1** includes inspection to determine the quality of packaging and labeling. The verification of a

counterfeit medicine is based primarily on a comparison between authentic and questioned products, involving detailed analysis of different elements in the existing packaging, drug leaflet and both the exterior and interior of the pharmaceutical dosage form (Jung, Ortiz, Limberger, & Mayorga, 2012). **Level 2** encompasses methods that can be done in the field. Failure during field screening should normally lead to submission to a quality assurance laboratory for validation. Occasionally more complex analysis may be required, in which case suspected products may be submitted to a forensic laboratory. **Level 3** requires the equipment of an established laboratory to determine drug quality according to established specifications (Kelesidis & Falagas, 2015).

Figure 2.3 Three stage process used in many countries for detecting counterfeits in field

2.5 Methods used to detect counterfeit medicines

Prevention strategies make use of a wide variety of technologies which are available to detect counterfeit and substandard medicines. Technologies used in the field for the detection of counterfeit medicines should be portable, relatively easy to use, inexpensive to buy and maintain and resistant in order to be used in developing countries with little financial capacity and where the detection process must be fast and there is little specialized personnel. They also must provide reliable and useful data.

Detection technologies provide varying degrees of qualitative and quantitative data about medicines. Qualitative techniques provide information about a medicine's identity, such as its active ingredient, color, or labeling. Quantitative techniques provide information about a medicine's content and how that content will be absorbed in the body. Qualitative assays may be used to quickly detect the least sophisticated falsified drugs, such as those with the wrong or no active ingredient. Quantitative deficiencies, such as an unacceptably high level of impurities or an unacceptably low or high dosage of active ingredient, are more common among substandard medicines. Tests for drug quality use both qualitative data (e.g., the identity of ingredients, the presence and nature of the packaging, the presence or absence of impurities, and any data referring to the medicine's appearance) and quantitative data (e.g., the amount of an ingredient present, tablet hardness, the rate and extent of disintegration and dissolution, and measured levels of impurities) (IoM, 2013).

The main categories of techniques for pharmaceutical analysis can be broken down as follows: visual inspection of product and packaging; tests for physical properties such as disintegration, reflectance spectroscopy, and refractive index; chemical tests including colorimetry and dissolution; chromatography; spectroscopic techniques; and mass spectrometry (MS) (Table 2.3). Within each of these categories, some technologies are appropriate for use in the field with minimal training, while others require sophisticated laboratory equipment and a high level of technical expertise (IoM, 2013).

There are also package technologies manufacturers may use to distinguish their products at the point of purchase. Holograms and reactive ink are examples of such package technologies. Regarding the analysis of the package, one could check for the presence of reactive ink on the packaging (that shows a text to be rubbed with metallic object), the existence of holographic security labels on packages, or recognize the print patterns on the package, including size and types of sources and figures. Such analysis is quite subjective because these technologies can be convincingly copied, and are clearly useless when the package of the original product is not available for comparison (Jung et al., 2012). As criminals become more sophisticated, there will be an increased need for expensive technologies to detect falsified medicines.

These analytical techniques which are available to detect counterfeit and substandard medicines are not capable of providing the necessary information to confirm the genuineness of a product when used individually. Therefore, the combination of techniques enables greater accuracy of the analysis. The combination of analytical techniques is a challenge for both field and laboratory since it is difficult to determine which tests must be combined so that the field inspectors use the minimum number of different techniques, in order to reduce the costs and period of analysis.

The costs associated with developing new detection technologies is a barrier to having robust, sustainable, easy-to-use, and inexpensive technologies available in the field. The question remains as to how to use analytical methods in parts of the world with limited laboratory capacity and trained chemists because the most sophisticated analytic technologies were not designed for the field (IoM, 2013).

Table 2.3 Methods used to detect counterfeit/substandard medicines adapted from (Kelesidis & Falagas, 2015)

Method	Advantage(s)	Disadvantage(s)	Reference(s)
Diagnostic methods to determine quality of medicines widely used in developing countries			
Inspection: physical properties (printing, embossing, shape, odor, taste, consistency) and secure labeling of products	● Quickest and cheapest way to detect a counterfeit medicine	● Neither sensitive nor specific since counterfeits have become increasingly sophisticated.	(Kelesidis & Falagas, 2015) (IoM, 2013)
Characteristic physical and chemical properties: weight, density, refractive index, viscosity, osmolarity, pH, crystal morphology, solubility	● Low cost ● Can provide simple tests for detecting counterfeit medicines	● Nonspecific	(Kelesidis & Falagas, 2015) (IoM, 2013)
Colorimetric techniques	● Can be done by untrained personnel ● Improved colorimetric methods incorporate colorimetric reactions onto a paper-based device ● Inexpensive	● Less sensitive and less specific than other more sophisticated techniques ● Gives limited information and destroys the sample under investigation	(Kelesidis & Falagas, 2015) (Green et al., 2015) (IoM, 2013)
Disintegration and Dissolution assays	● Widely used ● Correlate the in vivo bioavailability of an oral medicine to its in vitro solubility vs time profile	● Less sensitive and less specific than other more sophisticated techniques ● Dissolution tests require more training and sophisticated equipment	(Kelesidis & Falagas, 2015) (IoM, 2013) (Fernandez et al., 2011)
GPHF Mini-lab	● Reliable, simple, and inexpensive method	● Less sensitive and less specific than other, more sophisticated techniques ● Requires reagents, solvents, standards, training	("Global Pharma Health Fund (GPHF)," n.d.)
TLC	● In combination with colorimetric methods has been recognized by the WHO as appropriate technology for initial screening of a large number of drug samples in developing countries ● Fast, convenient, easy, inexpensive ● Useful in settings where laboratory resources such as HPLC are not available	● Often uses toxic or flammable reagents ● Provides limited information about a drug's identity ● Destroys the sample	(Kelesidis & Falagas, 2015) (IoM, 2013)
X-ray methods, such as X-ray fluorescence	● Can be used in resource-poor settings	● Only qualitative analysis of drugs ● Costly methods	(Kelesidis & Falagas, 2015)

	because they are portable		(Martino, Malet-Martino, Gilard, & Balayssac, 2010)
Spectroscopy methods	● Can be used in resource-poor settings because they are rapid, accurate, and can be performed with a portable device that allows real-time analysis of suspicious medicines ● Allows determination of a spectral print according to the drug ingredients and comparison with all spectra stored in a database	● High initial investment cost	(Kelesidis & Falagas, 2015) (IoM, 2013) (Dégardin, Guillemain, Viegas, & Roggo, 2016) (Dégardin et al., 2016)
CD3+	● Can be readily used by people without scientific or technical training ● Inexpensive tool ● Allows for the visual inspection of both the packaging and the dosage unit	● Not so good in the detection of the dosage unit as Raman spectroscopy or GPHF Mini-lab	(Batson et al., 2016) (Ranieri, 2013)
Diagnostic methods to determine quality of drugs that are not widely used in developing countries			
HPLC	● Determine the exact quantity of API in the sample ● Assess drug quality according to the specifications established by international pharmacopoeia ● Accurate identification of ingredients; used for nonvolatile chemicals	● Limited analysis speed ● Sophisticated, costly, labor-intensive ● It may not detect excipients that are not soluble in the mobile phase	(Kelesidis & Falagas, 2015) (IoM, 2013) (Martino et al., 2010)
Gas chromatography	● Useful for detecting residual solvents, volatile constituents, and undeclared ingredients	● Sophisticated, costly, labor-intensive ● It may only be used for separation of volatile materials	(Kelesidis & Falagas, 2015) (IoM, 2013)
MS methods	● Precise: accurate identification of ingredients present in counterfeit medicines ● Assess drug quality according to the specifications established by international pharmacopoeia ● No sample preparation	● Requires extensive training and expertise to use	(Kelesidis & Falagas, 2015) (IoM, 2013) (Fernandez et al., 2011)

Microbiological techniques	● Antimicrobial activity assays with reference bacterial species are specific	● Not widely available	(Kelesidis & Falagas, 2015) (Dieterpullirschagesat et al., 2014)
RFID system	● Label meant to receive a radio signal and immediately respond by sending back a different radio signal containing data; this label is made of an electronic chip and an antenna	● Very expensive implementation system	(Kelesidis & Falagas, 2015) (Taylor, 2014)

Abbreviations: API, active pharmaceutical ingredient; GPHF, German Pharma Health Fund; TLC, thin-layer chromatography; CD3+, Cluster of Differentiation; HPLC, high-performance liquid chromatography; MS, mass spectrometry; RFID, radio frequency identification.

An expert can identify some drug quality problems by sight. Therefore, **visual inspection** of a product and its packaging by someone who knows the properties of the authentic drug or is able to compare the sample to the authentic product is the standard first step in any medicine quality analysis. Differences from the authentic materials in color, size, shape, tablet quality, and packaging indicate a possible falsified or substandard drug. These differences range from subtle to obvious. Visual inspections are often unreliable because substandard and counterfeit medicines and their packaging often appear identical or very similar to the genuine products. Criminals who produce counterfeit medicines nowadays are able to copy holograms, bar codes, packaging styles, colors of tablets and markings with great precision (IoM, 2013).

Some tests that rely on pH and other **bulk properties** can help identify active ingredients. Bulk properties, also called intensive properties, are properties that do not depend on the amount of the chemical sampled. Density, solubility, reflectance spectra, refractive index, and optical rotation are examples of bulk properties (IoM, 2013). None of these techniques allows for a complete and specific analysis of the product under investigation but may be used in combination with other techniques to improve detection of counterfeit pharmaceuticals. For example refractive index can be used to measure the purity of pure liquids and can detect materials separated by liquid chromatography. Field inspectors can use handheld refractometers to measure the refractive index and use it as a quantitative test for some active ingredients (Fernandez et al., 2011).

A variety of simple chemical reactions can test for the presence of active ingredients. The **colorimetry** is one of those techniques. It relies on chemicals that undergo color changes when reacted with certain compounds to provide qualitative data about the identity of a medicament. Colorimetry protocols exist for the active ingredients in many essential medicines. In addition to verifying the presence of an active ingredient, colorimetry can serve as a semi-quantitative technique to provide information about tablet potency; a more drastic color change or deeper color generally indicates a larger amount of ingredient. Colorimetry gives limited information and

destroys the sample under investigation. However it is an inexpensive technique that does not require qualified personnel to work with it (IoM, 2013).

Disintegration and Dissolution testing may identify common formulation problems. Disintegration tests measure how rapidly solid dosage forms disintegrate in a solution; dissolution tests analyze the rates at which medicines dissolve. Dissolution tests require more training than colorimetry and disintegration testing but may help predict the bioavailability of medicines, an important aspect of their efficacy (IoM, 2013). The use of incorrect excipients, as well as inadequate manufacturing processes, may contribute to poor dissolution resulting in much lower or higher bioavailability, rendering these drugs substandard. Poor storage conditions resulting in decomposition products may also influence dissolution, but these samples should be considered "degraded" and not substandard (Fernandez et al., 2011). Disintegration tests are fairly simple and can be done in the field, but dissolution tests require sophisticated equipment (IoM, 2013).

The Global Pharma Health Fund (GPHF) set out to develop and supply inexpensive field test kits with simple test methods for rapid medicines quality verification and counterfeit medicines detection. Verifying the quality of medicines using the **GPHF-Minilab™** involves a three-stage test plan that employs very simple physical and chemical testing. Its starts with a physical inspection of dosage forms and associated packaging, then a simple tablet and capsule disintegration test and finally easy-to-use thin layer chromatographic tests for a quick check on drug content. Results obtained must match the product label claims for, at least, drug identity and content. If they do not match or results are inconclusive, then the appropriate batches can be frozen for further investigation in a laboratory. This method is less sensitive and less specific than other, more sophisticated techniques and requires reagents, solvents, standards, training ("Global Pharma Health Fund (GPHF)," n.d.).

Chromatography separates mixtures into their constituent parts based on a variety of chemical and physical properties. It can be used to separate drug ingredients for further testing and, when used with appropriate detectors, provides both qualitative and quantitative information about active ingredients and impurities. Like colorimetric tests, chromatographic analysis destroys the drug sample (IoM, 2013). Thin layer chromatography (TLC) and the closely related high performance thin layer chromatography (HPTLC) are members of a class planar chromatography where the separation is effected by solvent capillary action causing the mobile phase to move across the chromatographic plate. Planar chromatography offers very versatile possibilities for the detection of the materials separated. Detection with eyes includes direct observation of colored materials, visualization by spray reagent, UV lamps, iodine vapor staining, and oxidation by concentrated acids (Kaale, Risha, & Layloff, 2011).

TLC is an uncomplicated assay useful in developing countries because it yields "versatile and robust" results at a low cost. Compared to other chromatographic techniques such as HPLC, TLC

requires significantly less equipment and expertise. TLC solvents are often toxic or flammable, so these chemicals may be difficult to transport for field use. Furthermore, TLC provides limited information about a drug's identity; two samples that travel different distances are definitely not the same substance, but two different substances could appear identical using any chromatography technique if they are chemically similar enough. The inspector running the TLC assay must spot the plate correctly with the sample, which requires some training, and then compare the results to those obtained with reference standards (IoM, 2013).

HPLC is the most popular instrumental technique used for the analysis of pharmaceuticals (Martino et al., 2010). Depending on the associated detection technology, it can be expensive and require skilled operators and expensive, often scarce, solvents. The systems also require reliable electrical power, which can be an obstacle in developing countries (IoM, 2013). HPLC can identify and measure active ingredients and many impurities, but may not detect excipients that are not soluble in the mobile phase, hence it is necessary to use other analytical methods to define the complete chemical composition (expected and wrong APIs, impurities, excipients) of the medicines (Martino et al., 2010). It can be used with a variety of detection technologies such as MS and UV-visible spectroscopy (IoM, 2013).

Gas chromatography, the most powerful chromatographic technology, provides similar information as the other chromatography systems. However, it may only be used for separation of volatile materials, such as residual solvents, undeclared ingredients, and any volatile impurities. This technique can only be used when the compounds of interest are gaseous in the analytical temperature range and do not degrade at or before the assay's minimum temperature (IoM, 2013). Investigators can use gas chromatography to develop profiles of drugs' volatile impurities and use those profiles to link batches of medicines from the same source. This would be very useful because medicines with very similar impurity profiles may have the same origin (Mulligan, Brueggemeyer, Crockett, & Schepman, 1996).

Advanced chromatographic techniques (HPLC, and Gas Chromatography) require expensive equipment and maintenance. In addition, these tests can only be done in central laboratories and are therefore available only to countries which do not have many financial constraints. Thus, these methods are not as widely used once the most countries affected by counterfeit medicines are developing countries, with fewer facilities and economic capacity.

X-ray diffraction (XRD) and X-ray fluorescence are other techniques that can give substantial information about medicines contents. X-ray fluorescence is used for elemental analyses that can often distinguish real from falsified drugs (IoM, 2013). XRD, when new, fast diffraction techniques are used, is a suitable method for identification of differences in tablet coatings as well as, after coating removal, in drug composition. In fact, the diffraction patterns have many well-defined peaks, which can be used as fingerprint of a sample. XRD is therefore a reliable method to

discriminate fake from original medicines. However, it is insensitive and not quantitative (Martino et al., 2010).

Spectroscopy is a class of analytical techniques that measures the interaction of matter and electromagnetic radiation, thereby giving insight into chemical structure and contents. These techniques all provide qualitative data, and some provide significant quantitative data as well. Whenever a substance is excited by an energy source, it can absorb and emit radiation at certain wavelength, thereby allowing an observation of the substance behavior that will produce peaks often referred to as the chemical fingerprints. A chemist can extract detailed chemical and structural information from a spectrum, and an inspector with minimal training can also identify falsified and substandard medicines by comparing the drug spectra to reference materials in drug spectra databases (Fernandez et al., 2011). Thanks to chemometric tools, the chemical and physical signature of a suspect sample can be rapidly compared to the genuine references, providing a fast yes/no answer (Dégardin et al., 2016). These techniques are relatively straightforward to use and moderately expensive, and routine comparative applications do not require extensive training.

The US FDA developed **CD3+**, a counterfeit detection tool that is based on sample illumination at specific wavelengths of light and visual comparison of suspect sample and packaging materials to an authentic sample and it can be readily used by people without scientific or technical training (Ranieri, 2013). To test performance of the CD3+ in field conditions, a study regarding antimalarials was conducted in Ghana which compared the CD3+ side-by-side with two existing medicine quality screening technologies—TruScan™ Portable Raman spectrometer and GPHF Minilab®. Minilab is based on TLC and TruScan is based on Raman spectroscopy. The study revealed it to be effective in identifying counterfeit products and packaging but it is not as good in the detection of the dosage unit as Raman spectroscopy or TLC. Therefore the best option for CD3+ is to use it in combination with either TruScan or GPHF Minilab for complete examination of both the dosage units and the packaging to decide whether the product under investigation is authentic or counterfeit (Batson et al., 2016).

Mass spectrometry (MS) is a sophisticated analytical technique that requires extensive training and expertise to use. It can identify many active ingredients and excipients, as well as some impurities. There are different kinds of MS that can be used alone and in combination with other analyses to detect illegitimate drugs (IoM, 2013). Two of the most widely adopted MS techniques used for pharmaceutical analysis are Desorption Electrospray Ionazation (DESI) and Direct Analysis in Real Time (DART). Both allow investigating pharmaceuticals without any sample preparation, requiring only a few seconds per sample in their basic operation modes (Fernandez et al., 2011). DESI MS in particular provides information about tablet surface homogeneity and the distribution of active ingredients and excipients in or on the surface of a tablet. MS's ability to precisely measure molecular weight and compare fragmentation patterns can help distinguish

between compounds that differ by only one or two atoms. There are now portable machines that can take this sophisticated technology into the field. However, mass spectrometers require a stable electrical power source, which may be difficult to obtain in some developing countries (IoM, 2013).

Two potential threats arise from microbiological contaminations in pharmaceutical products. First, certain microorganisms may change the quality of the active ingredients. Second, microbiological contaminations may directly cause adverse effects to the patient by producing toxins or causing infections. Microbial contamination and infection are known to be serious risks associated with illegal drug use, the legal use of pharmaceuticals distributed under poor hygienic conditions, and counterfeit medicines for parenteral administration. **Antimicrobial activity assays** with reference bacterial species are useful for detecting counterfeit medicines because these medicines and the original medicines are not produced under the same hygienic conditions. However they are not used widely (Dieterpullirschagesat et al., 2014) (Kelesidis & Falagas, 2015).

The **RFID** system consists of a tag, a reader, and a computer and uses radio waves to transmit information between the three. RFID tags are passive, non-powered transmitters affixed to drug boxes or pill bottles with nearly unlimited lifespans (Taylor, 2014). Readers are radio frequency transmitters and receivers controlled via microprocessor that actively communicate with the tags. Readers use an antenna to capture data then pass it on to the computer for processing and storage. These systems can be instrumental in identifying counterfeit lots before they reach clinics and prevent thousands of adverse events that arise from counterfeit medicines every year. The larger challenges are economic and technical. Integrating RFID into a firm's existing Information Technology (IT) system is difficult and costly requiring either a strategic partnership with an Enterprise Resource Planning (ERP) firm or a significant investment in internal software (Taylor, 2014).

Chapter 3 - Raman Spectroscopy, a non-invasive measurement technique for the detection of counterfeit medicines

The following chapter provides a brief introduction to Raman spectroscopy. It includes an introduction to the Raman effect, instrumentation, spectral interpretation and data analysis. The continuous developments in Raman instrumentation have made it simple to use, and today Raman spectroscopy can be applied for analyzing many types of samples without having a deep knowledge of the Raman effect and features of the instrumentation. However some basic knowledge can be advantageous in assessing how suitable Raman spectroscopy is when analyzing a specific type of sample, what measurement conditions should be considered, and how spectra can be interpreted and analyzed. In this Chapter Raman spectroscopy is also compared to other spectroscopic techniques, It also includes a summary of studies regarding Raman spectroscopy for detecting counterfeit medicines.

3.1 Raman spectroscopy

3.1.1 Theorical basis

Raman Effect

The “Raman effect” was discovered by Chandrasekhara Venkata Raman, an Indian physician, together with Kariamanickam Srinivasa Krishnan. He first predicted and described the Raman effect as “A new type of secondary radiation”. In his study, he reported for the first time the inelastic scattering of light. Different light-scattering processes arise when photons of light interact with molecules in material (Brauchle & Schenke-Layland, 2013).

When light interacts with matter, it can be transmitted, absorbed or scattered. When light is scattered from a molecule, most photons are elastically scattered. The scattered photons therefore have the same energy, frequency and wavelength as the incident photons. However, a small fraction of light is inelastically scattered i.e. at frequencies different from, and usually lower than, the frequency of the incident photons. This is called Raman effect. Raman scattering can occur due to a change in vibrational, rotational or electronic energy of a molecule, but chemists are concerned primarily with the vibrational Raman effect that is why Raman effect usually means vibrational Raman effect only (Kaiser Optical Systems, 2004).

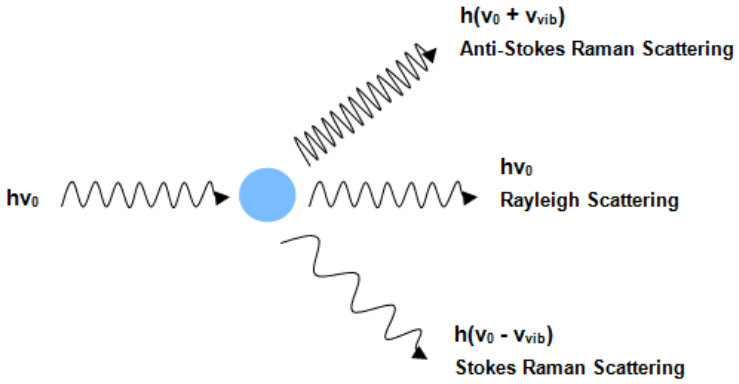

Figure 3.1 Light interaction with matter giving rise to elastically scattered photons with the same energy (frequency) and to a small fraction of inelastically scattered photons with different energy (frequency) - Stokes and Anti-Stokes scattering. Adapted from (Lotte Boge Lyndgaard, 2013)

Photons can be imagined to undergo a collision with molecules (Figure 3.1). When light incidents on a molecule, it can interact with the molecule but the exchange of energy is zero, so the frequency of the scattered light is the same as that of the incident light (v0). This process is known as Rayleigh scattering (Das & Agrawal, 2011). If the interaction causes the light photon to gain vibrational energy from the molecule then the frequency of the scattered light will be higher than that of the incident light ($v_0 + v_{vib}$), known as Anti-Stokes Raman scattering. If the interaction causes the molecule to gain energy from the photon then the frequency of the scattered light will be lower than that of the incident light ($v_0 - v_{vib}$), this process is known as Stokes Raman scattering (Das & Agrawal, 2011).
A plot of intensity of scattered light versus energy difference, then, is a Raman spectrum (Kaiser Optical Systems, 2004).

The Raman effect arises when a photon is incident on a molecule and interacts with the electric dipole of the molecule. Hence, the interaction can be viewed as a perturbation of the molecule's electric field causing the electrons of the molecule to polarize (being distorted in an opposite direction compared to the nuclei) and go to a higher "virtual" energy level (Figure 3.2) (Lotte Boge Lyndgaard, 2013). As C.V. Raman and K.S. Krishnan said: the "(…) scattering of the *unmodified* type (…) corresponds to the normal or average state of the atoms and molecules, while the *modified* scattering of altered wavelength corresponds to their fluctuations from that state,(…)" (RAMAN & KRISHNAN, 1928). The scattering event occurs in 10^{-14} seconds or less. The difference in energy between the incident photon and the Raman scattered photon is equal to the energy of a vibration of the scattering molecule (Kaiser Optical Systems, 2004).

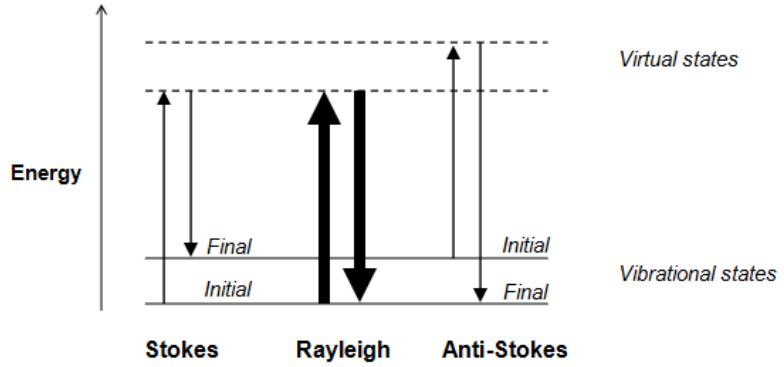

Figure 3.2 Energy level diagram for Rayleigh and Raman scattering processes. Modified from (Kaiser Optical Systems, 2004)

The energy difference between the incident and scattered photons is represented by the arrows of different lengths in Figure 3.2. Numerically, the energy difference between the initial and final vibrational states, v, or Raman shift in wavenumbers (cm^{-1}), is calculated through equation 1:

$$\bar{v} = \left(\frac{1}{\lambda_{incident}} - \frac{1}{\lambda_{scattered}}\right) \times 10^7 \qquad (1)$$

in which $\lambda_{incident}$ and $\lambda_{scattered}$ are the wavelengths (in nm) of the incident and Raman scattered photons, respectively. It is important to note that the Raman shift is independent of the incident laser frequency. The vibrational energy is ultimately dissipated as heat. Because of the low intensity of Raman scattering, the heat dissipation does not cause a measurable temperature rise in a material (Lotte Boge Lyndgaard, 2013).

The Raman effect is weak. Only approximately one out of 10^8 incident photons is shifted. Even fewer are anti-Stokes scattered because the majority of molecules are in the ground vibrational energy state at room temperature, and not in the excited state as required for generating anti-Stokes scattering (Figure 3.2) (Lotte Boge Lyndgaard, 2013). The Stokes and anti-Stokes spectra contain the same frequency information. The ratio of anti-Stokes to Stokes intensity at any vibrational frequency is a measure of temperature (Kaiser Optical Systems, 2004).

Polarizability

Above was mentioned the polarization of the electrons in one molecule. To understand this concept it is important to talk about the dipole moment of a molecule. It is important to absorb and scatter light. The dipole moment is the center of the positive and the negative charges, where molecules that possess a permanent dipole moment are said to be polar. Even if there is no permanent dipole moment, it is possible to induce one by the application of an external electric field. The dipole moment is induced because the electric field can change the distribution of the electrons slightly within the molecule, by pushing the negative electrons and the positive nuclei in

opposite directions. This is called polarization, and the change of the dipole moment induced is a measure of the polarizability of the molecular species (Lotte Boge Lyndgaard, 2013).

A simple electromagnetic field description of Raman spectroscopy can be used to explain many of the important features of Raman band intensities. The dipole moment, **P**, induced in a molecule by an external electric field, **E**, is proportional to the field as shown in equation 2, where α is the polarizability of a molecule (Kaiser Optical Systems, 2004).

$$\mathbf{P} = \alpha \mathbf{E} \qquad (2)$$

In other words the magnitude of the induced dipole moment depends both on the magnitude of the applied field (the intensity of the laser light) and the ease with which the electron cloud around the molecule can be distorted. Raman scattering occur because of molecular vibrations that change the polarizability of the molecule. The change is described by the polarizability derivative, $\frac{\delta\alpha}{\delta Q}$, where **Q** is the normal coordinate of the vibration.

As the strength of the scattered light is proportional to the electron cloud displacement, molecular vibrations can cause a periodic change in the intensity of the scattered light. The position of the displacement is equal to the energy of the chemical bond that has been polarized. The energy can be added to or subtracted from the original frequency of the light. If we define the (virtual) normal coordinates Q to fix the position of a molecule in the three dimensional space, Q will change during molecular vibration and will thus be given by the derivative δQ.

The selection rule for a Raman active vibration, that there be a change in polarizability during the vibration, is given in equation $\frac{\delta\alpha}{\delta Q} \neq 0$. Scattering intensity is proportional to the square of the induced dipole moment, that is, to the square of the polarizability derivative $\left(\frac{\delta\alpha}{\delta Q}\right)^2$ (Lotte Boge Lyndgaard, 2013).

If a vibration does not greatly change the polarizability, then the polarizability derivative will be near zero, and the intensity of the Raman band will be absent. The vibrations of a highly polar bond are usually weak. An external electric field cannot induce a large change in the dipole moment, and stretching or bending the bond does not change this. Typical strong Raman scatterers are double bonds such as carbon-carbon bonds. In this case bending or stretching the bond changes the distribution of electron density substantially and causes a large change in induced dipole moment. The observed strength of the Raman band is also proportional to the concentration of the species, as well as the intensity of the excitation laser (Kaiser Optical Systems, 2004).

Molecular Vibrations

Raman spectroscopy is used to obtain information about structure and properties of molecules from their vibrational transitions. In vibrational spectroscopy this molecular property is utilized by studying the interactions between light and these molecular vibrations. The energy of most molecular vibrations corresponds to that of the infrared (IR) region of the electromagnetic spectrum, which falls between 4000cm^{-1} and 200cm^{-1}. Vibrational energy for a molecular bond mainly depends on the mass of the atoms involved and the strength of the covalent bond.

The stronger the bond between two atoms is, the higher the vibrational frequency. For triple bonds the bond strength is higher than for double bonds resulting in a higher vibrational frequency for triple bonds than double bonds. Conversely, the heavier the vibrating masses, the lower the vibrational frequency. Thus, the vibrational frequency for bonds involving hydrogen has the highest frequency because hydrogen has a small mass. The concept of bond vibration in not very exact. For each vibration, all atoms in a molecule vibrate, but they can be more or less localized. The X-H vibrations are very localized, so are the triple bonds, but for double bonds in for example peptides, they become less localized and so forth (Lotte Boge Lyndgaard, 2013).

3.1.2 Utility and Limitations of Raman Spectroscopy

The intensity of Raman scattered light is proportional to the number of molecules that produce the Raman scattered light. As a result, the intensity of Raman scattered light can be used to measure how much of a material is in a sample (quantitative analysis). The shape of a Raman spectrum can be used to determine the types of molecular vibrations in a sample. This vibrational information can be used to identify materials in a sample (qualitative analysis). Differences in stress, temperature, crystal structure, micro-heterogeneity, etc. can, therefore, often be measured using Raman spectroscopy (Kaiser Optical Systems, 2004). Raman spectroscopy is beneficial for diverse types of quantitative and qualitative analyses in a vast number of fields, including fundamental chemical research, life sciences (in vivo biomedical studies), process control, forensic sciences, and pharmaceuticals (Rojalin et al., 2016) (Brauchle & Schenke-Layland, 2013). It can be employed as a non-invasive, non-destructive, and even non-contact monitoring technology (Brauchle & Schenke-Layland, 2013).

Raman spectroscopy can be used to monitor chemical reactions. The disappearance of starting materials and the appearance of products cause easily predictable changes in the Raman spectrum. These changes can be used to determine how close the reaction is to completion. The Raman measurement can be made by simply pointing a probe at the glassware containing the chemical reaction. Nothing needs to be removed from or added to the standard reaction vessel. Data collection and result presentation can be completed in as little as a few seconds (Kaiser Optical Systems, 2004).

Raman spectroscopy has been applied to a wide range of other measurement problems. Residual monomer concentration in polymer products, polymorphism in pharmaceutical products, and crystallinity in semiconductors and polymers are just a few of the practical examples. Raman spectroscopy also appears promising for the non-destructive measurement of packaged materials without removing the materials from the package (Kaiser Optical Systems, 2004).
The analytical versatility of Raman molecular spectroscopic techniques allows for investigating a wide diversity of transparent, translucent, opaque, and colored samples including solids, semi-solids, suspensions, and solutions. This facilitates non-invasive analysis of synthetic reaction mixtures, biological specimens, and intermediates to various finished dosage forms (such as powders, tablet/capsule, creams, heterogeneous suspensions, and syrups) with minimal or no sample preparations (Paudel et al., 2015).

An area of particular interest and high relevance to pharmaceutical applications is the quantification or identification of specific polymorphic forms of an API in a final dosage form. The necessity to quantify polymorphic forms often falls into two main areas. Firstly, from a commercial stand-point e.g. patent infringement, a patent may protect only one particular drug form. Secondly, efficacy assurance since the solubility (a function of polymorphic form) of the specific drug form will affect the bioavailability of the API (Griffen, Owen, Burley, Taresco, & Matousek, 2016).
Summarizing the known advantages of Raman spectroscopy include: high chemical specificity; the ability to quantify multiple constituents of a solid dose form, the ability to analyse polymorphs and crystalline state; the high speed of analysis; the absence of sample preparation; the absence of solvents and/or consumables and the non-destructive nature of analysis compared with the traditional analytical techniques (Griffen et al., 2016). Overall, Raman spectroscopy is an established laser-based technology for the quality assurance of pharmaceutical products (Brauchle & Schenke-Layland, 2013). It can readily identify many active ingredients and give further information about excipients, as well as the relative concentration of active ingredients to excipients. These ratios can be key to detecting falsified and substandard drugs, because criminal manufacturers often take care to use the correct amount of active ingredient but may not be as exacting about the excipients, which may vary even among genuine manufacturers (IoM, 2013).

Major limitations to Raman spectroscopy use have included photoluminescence frequently masking the Raman signal, weak Raman scattering, and insufficiently sensitive detectors (Rojalin et al., 2016).

Sensitivity

Raman spectroscopy is not a very sensitive technique. The limited sensitivity is a direct consequence of the low probability for the detection of a Raman scattered photon. Typically, more than 100 000 000 photons of incident light on the sample are needed to produce a single Raman scattered photon from a pure sample (100% concentration). Raman photons are scattered in nearly all directions, so only a fraction of them can be collected by the Raman instrument. Optical

processing of the collected Raman photons by traditional Raman instruments loses 80% to 97% of the photons. Photomultipliers, the traditional light-measuring devices in Raman instruments, detect only 1 photon out of 5 (or worse depending on the frequency of the light). Reliable measurements require many detected Raman photons, so low material concentrations are difficult to measure using Raman spectroscopy (Kaiser Optical Systems, 2004).

Background

In addition to Raman scattered light, most samples produce other types of scattered light when illuminated. The contribution from this "non-Raman light" can usually be subtracted from the Raman spectrum, but the noise from the non-Raman light cannot. The minimum noise in an optical intensity measurement is equal to the square root of the number of detected photons due to the statistical nature of light. This minimum noise is called *shot noise*.

When the *shot noise* from the background is too large compared to the intensity of the Raman signal, the Raman measurement cannot be reliably made. The most common source of background encountered in Raman measurements is fluorescence. Fluorescence is a much more efficient process than Raman scattering. A single incident photon can produce a fluorescence photon with a high probability. As a result, impurities at the parts-per-million level that fluoresce can obscure the Raman spectrum of an otherwise pure material (Kaiser Optical Systems, 2004).

Some of these problems have been resolved with improvements in hardware, such as the development of efficient, low-noise, detectors made of silicon, stable high-power diode lasers, and high resolution spectrometers. This way, Raman has become widely applied in both basic and applied chemistry. Despite the significant technical advances, the weak signal and simultaneously strongly interference ring background from photoluminescence are still hindrances in Raman spectroscopy (Rojalin et al., 2016).

3.1.3 Raman Spectroscopy compared to other spectroscopic techniques

Spectroscopic methods can be based on phenomena of emission, absorption, fluorescence or scattering. Different spectroscopic methods are frequently used for the characterization of a wide range of samples (Bumbrah & Sharma, 2016).

When choosing the most appropriate analytical method for a given problem, a number of practical and theoretical properties must be considered. These properties are related to the performance of spectroscopic techniques regarding to their applicability for a rapid quality control and/or process monitoring. The characteristics of the spectroscopic method must be balanced taking into consideration the molecular properties of the analyte so that the right choice is made.

Table 3.1 provides the essential information to be taken into account when choosing a spectroscopic method, for five techniques frequently used, considering its multivariate information

contents. These methods are based on different regions of the electromagnetic spectrum, on different physical principles and have different sensing capabilities. The properties to take into account when choosing the right spectroscopic technique are: spectral resolution, sensitivity, selectivity, the amount of interferences, the ease of sample preparation, sampling flexibility, typical analysis time and financial costs.

The **spectral resolution** can have an influence on the ability to distinguish between analytes. NIR, UV-VIS and fluorescence typically have low spectral resolution whereas IR and Raman spectroscopy have high spectral resolution. Chemometrics can, however, often be used to overcome problems of low spectral resolution by resolving the chemical information mathematically.

Sensitivity is about the information content. The method must be sensitive enough to measure the analyte or quality parameter of interest and this depends on the molecular properties of the analyte. For example, in non-resonance Raman spectroscopy the change in polarizability during a molecular vibration is measured. Thus, the Raman signal is large for vibrations that do not change the symmetry of a molecule, vibrations of C-C double and triple bonds and for molecules containing S, Cl or Br. Surface-enhanced Raman spectroscopy (SERS) has drawn widely attention in the field of chemistry, biology and material science due to its abundant molecular fingerprint information and high detection sensitivity, which can serve as a useful method for rapid analysis of various analytes (Wang et al., 2015). IR spectroscopy is based on absorbance of IR radiation by fundamental molecular vibrations with a permanent dipole moment or an induced dipole moment during vibration. The C=O stretch gives particular large absorbance in IR. NIR is especially sensitive to strong anharmonic vibrations which are typical for X-H bond vibrations where X is a heavier atom. This NIR selection criterion gives preference to C-H, N-H and O-H vibrations which are abundant in biological systems for example. Absorption of UV-VIS radiation causes valence electron transitions in molecules enabling measurement of analyte down to very low concentrations (ppm range).For fluorophore molecules the analytes ca be measured to even lower concentrations (ppb) (Lotte Boge Lyndgaard, 2013).

Selectivity plays an important role in the ease of calibration. Quantification by Raman spectroscopy can be achieved with a small to modest set of reference values by selection of the analyte specific spectral bands or regions; the same can be anticipated for IR and fluorescence spectroscopy applications (assuming that the analyte of interest is a fluorophore for the last one). This is not the case for NIR or UV-VIS which present low selectivity, leading to larger investments for quantification.

The **amount of interference** is also important, as it may cover/interfere with the signal of the analyte of interest, and thereby give a biased result. For Raman spectroscopy the major

interference is fluorescence, whereas for NIR and specially IR water can interfere and cover the signal of interest (Lotte Boge Lyndgaard, 2013).

The choice of spectroscopic method also depends on the requirements regarding the ease of sampling requirements such as **sample preparation**, **sampling flexibility** and **analysis time**. NIR has been the primary choice in many industrial applications as it is fast, requires no sample preparation and can measure remotely and non-invasively. Raman possesses many of the same properties and can sometimes measure analytes which NIR cannot determine. However, Raman probes must be shielded from external lights (room light for example) during the measurement (Lotte Boge Lyndgaard, 2013). Recent developments of portable near-infrared and Raman spectrometers have led to an increase in the use of these techniques for drug quality analysis. Both techniques are nondestructive, fast, and require no sample preparation and radiation can pass through samples in blister packs (IoM, 2013). For IR the possibilities of using optical fibers are very restricted, thereby decreasing the sampling flexibility (i.e. making on-line and in-line installations limited). For both UV-VIS and fluorescence optical fiber probes are available, making *in-situ* process sampling possible (Lotte Boge Lyndgaard, 2013).

Concerning financial costs, Raman installations are the ones that require greater investment. For an *in-line* application installation costs are between two to five times the purchase costs and this ratio can be much higher for a Raman installation due to laser-related safety precautions. Also the maintenance costs of the laser in Raman spectroscopy can be a disadvantage compared to light sources used in NIR or IR. However, these cost-disadvantages might be offset by the ease of calibration mentioned before and the confidence Raman measurement can bring (e.g. the clearly recognizable spectral band(s) uniquely associate with the analyte of interest) and also the good stability and reproducibility for measurements in practice (Li et al., 2016).

Table 3.1 Comparison of properties of five spectroscopic methods frequently used for multivariate analysis. Adapted from (Lotte Boge Lyndgaard, 2013)

	NIR absorption	**Raman scattering**	**IR absorption**	**UV-VIS absorption**	**Fluorescence emission**
Resolution	Low	High	High	Low	Medium
Sensitivity	Low	Medium	Medium	High	High
Selectivity	Medium	High	High	Low	Very High
"Main Interferences"	Water	Fluorescence	Water	Scattering	Quenching
Sample preparation	None	None	None	None	None
Sampling flexibility	High	High	Low	Medium	Medium
Typical analysis time	Seconds	Seconds	Minutes	Seconds	Minutes
Financial Costs	++	++++	++	+	+++

Other methods, such as Fourier transform infrared (FTIR) spectroscopy and Nuclear magnetic resonance (NMR) spectroscopy can often provide the same information as Raman spectroscopy. Raman measurements, however, usually require less sample preparation, and can often be done remotely without modifying the sample or even removing it from its packaging. This makes Raman spectroscopy a method of choice for real-time process monitoring and non-destructive testing. In addition, Raman measurements can be made on very small samples (Kaiser Optical Systems, 2004). Another advantage is the variety of robust mini and portable Raman systems that have become available commercially at increasingly competitive prices (Nieuwoudt, Holroyd, Mcgoverin, Simpson, & Williams, 2016).

3.1.4 Raman Instrumentation and configurations

Instrumental evolutions of Raman-based techniques have progressed from traditional laboratory spectrometers to a wide range of miniaturized and tunable lasers, optical filters, spectrographs, interferometers, charge-coupled device (CCD), microprocessor control and Raman probes. Today, Raman-based tools are cheaper, smaller, smarter and faster, and the analysis of “real world samples” in-line from production lines or inside the packaged containers has gone from a concept idea to a well-established practice for different pharmaceutical products. This has led to the recognition of Raman spectroscopy by regulatory authorities for innovative analysis (Paudel et al., 2015).

In Raman spectroscopy, the sample is illuminated with a monochromatic laser beam which interacts with the molecules of the sample and originates a scattered light. These laser sources provide stable and intense beam of radiation. Due to its properties, lasers are almost exclusively used for excitation. Wide range of lasers such as Argon ion laser (which can excite at 488 nm and 514.5 nm), Krypton ion laser (530.9 nm and 647.1 nm), Helium–Neon (He–Ne) (632.8 nm), Near Infrared diode lasers (785 and 830 nm), Neodymium–Yttrium Aluminum Garnet (Nd:YAG) and Neodymium–Yttrium Ortho-Vanadate (Nd:YVO4) (1064 nm) and frequency doubled Nd:YAG and Nd:YVO4 diode lasers (532 nm) can be used as light source in Raman spectrophotometers (Bumbrah & Sharma, 2016).

The choice of laser is critical to the success of Raman for a given application and has major effects on both the observed spectrum and the instrument design. Short wavelength sources such as Argon ion and Krypton ion lasers can produce significant fluorescence and cause photodecomposition of the sample However, a short wavelength can be detected with less noise and the number of Raman photons available for detection is much higher than if a longer wavelength laser is applied, resulting in an improved sensitivity. Long wavelength sources such as diode or Nd:YAG lasers can be operated at much higher power without causing photodecomposition of sample and eliminates or reduces fluorescence in most cases. Usually, as longer wavelengths are applied, the background tends to decrease (Bumbrah & Sharma,

2016). Therefore, the laser wavelength should be as long as required to avoid fluorescence, but not so long that the signal strength and detector noise become problematic.

Raman instruments require a laser-line rejection device to filter out the reflected laser radiation and Rayleigh scatter from the much weaker Raman scattering. The most common choice is the holographic notch filter where a narrow band of wavelengths is reflected, while all other wavelengths are transmitted with high efficiency (Lotte Boge Lyndgaard, 2013).

All Raman instruments include a laser, an interface between sample and instrument, a filter to remove Rayleigh scatter, a spectrograph to separate the Raman scattered light by wavelength, a detector and a communication system to report analysis results (Lotte Boge Lyndgaard, 2013). Raman spectrometers can be dispersive or nondispersive. **Dispersive Raman spectrometer** use prism or grating which disperses light into a charge-coupled device (CCD) while **non-dispersive Raman spectrometer** uses an interferometer in Fourier Transform Raman spectrometer (Bumbrah & Sharma, 2016).

In a **dispersive Raman spectrometer**, presented in Figure 3.3, the monochromatic light from the laser is coupled into an optical fiber bundle. The fiber bundle carries the laser excitation to the probe. Silica Raman scattering and fluorescence generated in the collection fiber is filtered out at the probe head, and the laser light is focused onto the sample. Backscatter from the sample is filtered to remove the light at the laser wavelength (Rayleigh scattering), collected, focused and sent to the base unit of the Raman instrument through another fiber bundle. In the base unit a holographic notch filter is used to remove any residual laser light (Rayleigh) and transmits the Raman scattered light to a holographic transmission grating. The grating disperses and diffracts the transmitted light and projects it into the CCD camera via lenses. CCD converts the incoming optical signal into charge. The accumulated charge on the pixels of the CCD camera is read out and converted into a Raman spectrum (Lotte Boge Lyndgaard, 2013).

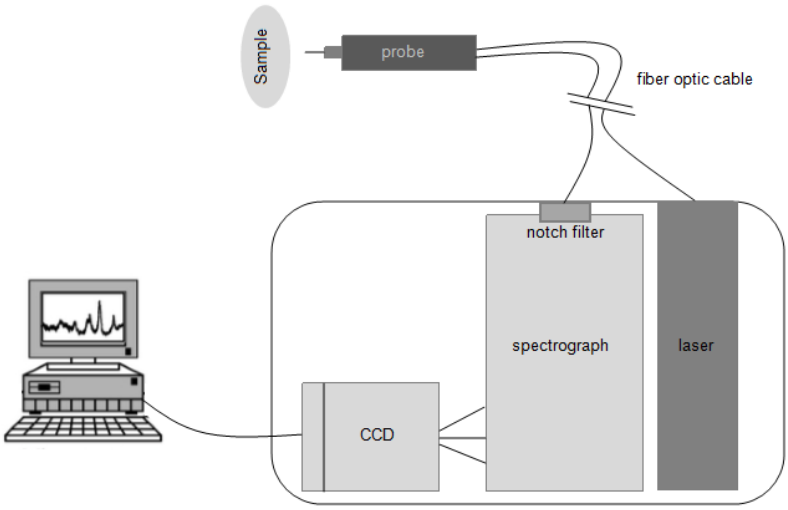

Figure 3.3 An overview of the elements of a dispersive CCD Raman instrument. Modified from (Lotte Boge Lyndgaard, 2013)

A CCD detector is made of silicon or other photosensitive semiconductors and registers the Raman signal. The CCD camera consists of two-dimensional arrays of pixels that each can be considered as an independent detector. The horizontal arrays of pixels are calibrated to correspond to the wavenumber axis, and the vertical pixels measure the intensity of Raman signal. The image on the CCD camera is an electronic picture of Raman signal, which is then converted into a spectrum. In most Raman instruments, the CCD is cooled below room temperature (-40ºC) in order to improve sensitivity of the detector system. Sensitivity of a detector is defined as the probability that a photon reaching the detector generates an electronically measurable signal, usually a photoelectron (Lotte Boge Lyndgaard, 2013).

Commercial **Fourier Transform-Raman spectrometers** (FT-Raman) were introduced in late 1980's to improve the detection system capable of overcoming the limitations of CCD and other detectors for operating in the near-IR region when using 1064 nm laser excitation. CCD detectors are insensitive for laser wavelength excitation above ~1050 nm, which means that for non-dispersive instruments where a 1064 nm laser is used, a noisier detector has to be applied. FT-Raman spectrometer uses an interferometer and continuous wave laser such as Nd:YAG. The current detectors of choice for Raman measurements at wavelength longer than 1000 nm are indium gallium arsenide (InGaAs) and germanium (Ge) detectors. These detectors are sometimes operated at cryogenic temperatures in order to reduce noise. Since water absorbs in the 1000 nm region, aqueous samples cannot be analyzed by FT-Raman spectrometer (Bumbrah & Sharma, 2016).

Interface between sample and instrument – Probes

The optical interface in the Raman instrument illuminates the sample with laser light, collects the Raman scatter from the sample material, and directs it to the spectrograph. Due to remote sensing and flexibility, the fiber-coupled sampling probes have been preferred in the recent years. Most Raman fiber optics probes use 180º backscatter geometry, which refers to the angle between the sample and the collection optics. The light is delivered to the sample by a single fiber, and the 180º scattered Raman signal is collected through another fiber or bundle of fibers (Lotte Boge Lyndgaard, 2013).

The incorporation of short wavelength lasers in Raman spectrophotometers opens the doors for use of telecommunications-type optical fibers such as remote-fiber-optic probes which can be operated over long distances (>10 m in some instances) and are well suited for in-situ or on-site analysis of samples. These fiber optic probes can also be used to record the Raman spectra in locations remote from the sample site and thereby prevent the exposure of investigator to hazardous environment (Bumbrah & Sharma, 2016).

In the selection of the Raman probe it is crucial to consider the nature of the sample being measured (e.g. phase, density and transparency/opacity). Furthermore, probe functionalities such as the confocality and the spot size are also very important. The **confocality** is a measure of how localized/focused the signal collection is in relation to the sample. The confocality for some probes is designed with a long focus making it possible to measure a sample through a process window, and only obtain data from the sample within. The **laser spot size** is determining for the sampling volume and can range from a few µm up to 6 mm (Lotte Boge Lyndgaard, 2013).

Probes ideal for **large volume samples** include wide-area-illumination (WAI) and spatially offset Raman spectroscopy (SORS) probes, and transmission set-ups. A traditional Raman analysis acquires data from a small spot of an analyte. Therefore, the resulting spectral output may fail to entirely represent the static and heterogeneous sample. Sample rotation during spectral acquisition and the temporal averaging of the acquired data, the spatial averaging of the data acquired by scanning different regions of sample, and simultaneous wide-angle illumination (WAI) are configurations available to overcome the issues related to sub-sampling (Figure 3.4) (Online, 2013).

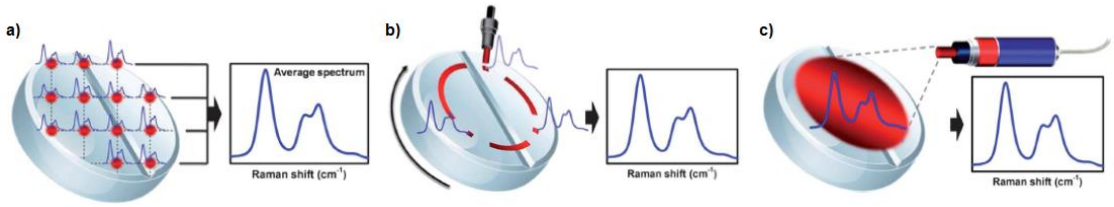

Figure 3.4 Three major schemes to improve sample representation through wide coverage of sample: a) averaging of Raman spectra collected at many different spots on a sample, b) rotation of a sample during spectral collection and c) simultaneous wide area illumination for spectral collection. Adapted from (Online, 2013)

Using WAI probe it is possible to measure with a spot size diameter of 3-6 mm. A dispersive Raman probe constituting multiple optical fibers, known as PhAT (Pharmaceutical Area Testing), measures a significantly large sample volume (Figure 3.5) (Paudel et al., 2015). The wide spot size allows a much greater portion of a sample to be interrogated in a single measurement than with a small sample volume probe. The WAI approach reduces the need for multiple measurement points and can therefore reduce the analysis time. However, a major drawback with many WAI probes is that they are limited only to measure a frequency range from 1800 cm^{-1} to 200 cm^{-1} (Lotte Boge Lyndgaard, 2013).

Figure 3.5 The PhAT probe from Kaiser represents the in situ approach to Raman solids sampling. Solids sampling by Raman is prone to under-sampling. This problem is solved using the WAI approach, which provides reproducible, representative, focus-free, quantitative Raman measurements ("Kaiser, optical systems, inc.," n.d.)

The configuration enabling the collection of signals from locations laterally offset away (hundred micrometers to centimeters in some cases) from the illuminated area is called SORS. Typical SORS geometry irradiates the laser at the center of the ring and Raman collection from the circumference, the radius being the spatial offset (Figure 3.6) (Paudel et al., 2015).

Spectral acquisition representing a bulk sample can also be performed using Transmission Raman Spectroscopy (TRS), wherein the incident and the collection beam path are separated to the extreme on opposite sides of sample. TRS potentially avoids the sub-sampling problem of

heterogeneous samples and yields (semi) averaged spectral data of the bulk composition for turbid or opaque materials (Figure 3.6) (Paudel et al., 2015).

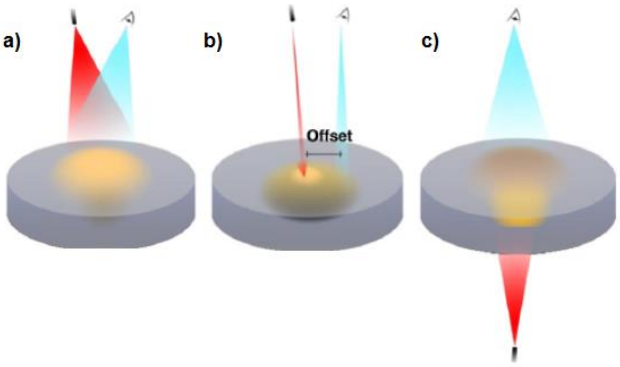

Figure 3.6 Different Raman spectroscopic configurations: a) WAI, b) SORS and c) TRS. Adapted from (Paudel et al., 2015)

Small volume sampling approaches often include optical microscopes. In Raman micro spectroscopy, Raman spectrophotometer is interfaced to an optical microscope which enables both visual and spectroscopic examinations as either as single point, mapping or imaging measurements. Microscope is used to focus the laser beam onto the sample. This set-up enables the visual inspection of sample through microscope objectives and facilitates spectroscopic analysis of a determined region within the sample. Measurements with high spatial resolution can be obtained and Raman images can be generated through rapid scanning of a sample stage originating a map with the spatial distribution of the sample's compounds. For samples where it is interesting to know the distribution of multiple compounds over a small area, this set-up is ideal (sometimes it may be enough to do multiple point scans or a line instead of a full mapping) (Bumbrah & Sharma, 2016). Regarding the applicability of Raman micro spectroscopy Figure 3.7 presents Raman imaging measurements of three tablets, where one can observe two different things: 1) the difference between two mixing processes performed well (a) and b)) using adequate containers with glass spheres on movement for a good homogeneity, and a mixture in which glass spheres were not present to simulate a poor homogeneity of the mixture (c)); 2) The difference between the imaging of a tablet with low concentrations of paracetamol (a)) and a tablet with a high concentration of paracetamol (b)). The preparation method of tablets is described in the Materials and Methods section of this dissertation.

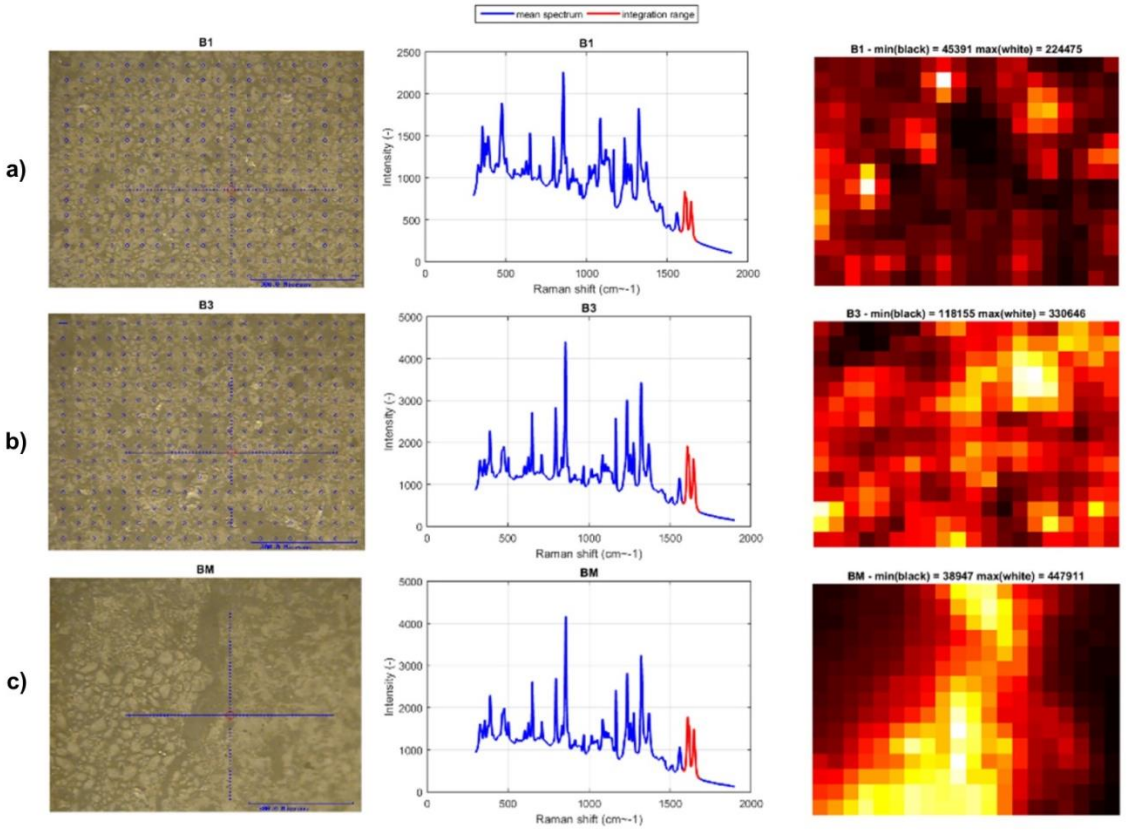

Figure 3.7 Microscopic imaging measurements obtained from Raman microprobe combined with MK II probe from Kaiser Optical Systems. a) Imaging of a tablet from Blend 1 (50mg of Paracetamol), b) imaging of a tablet from Blend 3 (150mg of Paracetamol) and c) imaging of a tablet with a Bad Mixing process.

There are also **immersion probes** which are robust devices that can be mounted directly into a process stream, reaction vessel, etc. It uses only a single fiber for excitation and another for collection. This probe can be immersed directly into a fermenter, and continues measurements can be carried out during the entire fermentation. The fermenter should be fully covered to avoid spectral interference from the room light. This way, fermentations may be monitored in real time (Lotte Boge Lyndgaard, 2013).

Low sensitivity due to weak Raman scattering is the major problem associated conventional Raman Spectroscopy. Therefore there are other techniques which can enhance sensitivity such as Resonance Raman Spectroscopy (**RRS)** and Surface Enhanced Raman Spectroscopy (**SERS**). In RRS, frequency of incident radiation matches with an electronic transition of molecule and as a result of this match, much more intense Raman spectrum is obtained. SERS is a modified technique in which sample is adsorbed on a colloidal metallic surface (silver, gold or copper) and thereby improves the intensity of Raman signals and also quenches the fluorescence caused by cutting agents, diluents and matrices. The combination of RRS and SERS techniques (i.e. Surface Enhanced Resonance Raman spectroscopy (SERRS)) can amplify the sensitivity up to ten orders of magnitude as compared to conventional Raman spectroscopy (Bumbrah & Sharma, 2016).

Thanks to the recent increasing ubiquity and portability of compact Raman spectrometers, the spectrometers are now more amenable to integration with many other analytical tools, thus enabling *in-situ* spectroscopy. The simplicity and compactness of handheld and portable Raman devices will further increase their application for anti-counterfeiting and quality control purposes (Paudel et al., 2015). Another measure that would facilitate the use of Raman spectroscopy in the detection of counterfeit medicines would be the expansion of the Raman active ingredient **database**. All drug detection technologies would be more powerful if there were a full authentication database with information about drug color, shape, size, weight, Raman and near-infrared reflectance. Drug companies resist against releasing this information, but stringent regulatory agencies should require it. Sharing all drug authentication information in a drug quality library would improve the power of existing drug detection technologies (IoM, 2013).

Acquisition Parameters

When the right sample interface is selected for a measurement there are some parameters that can be turned or selected prior to acquisition (Lotte Boge Lyndgaard, 2013). The main **acquisition parameters** are:

- Exposure – it corresponds to the time where the spectrally dispersed light emerging from the spectrometer is allowed to strike the pixels of the CCD array and accumulate charge. In most dispersive Raman systems, a mechanical shutter is used. When not exposing the shutter prevent light from striking the CCD detector. Between exposures the detector may be set to do a cleaning, where any charge that is accumulated in the pixels is flushed from the CCD.
- Accumulation – it is a technique for very effective reduction of random noise inherent in the Raman spectrum and it is related to the number of spectra accumulated.
- Dark subtraction – it is typically carried out by collecting a dark spectrum i.e. an exposure with the camera shutter closed, and saving that spectrum for subtraction from all subsequently spectra to be recorded under similar conditions. A new dark spectrum must be acquired whenever the acquisition parameters are changed.

3.1.5 Raman Data Analysis

Spectrum

A Raman spectrum often contains a large amount of information that describes the chemical composition and the physical properties of the sample, but also unwanted non-sample information originating from noise and external light.

A Raman spectrum is presented as an intensity versus wavelength shift. Raman spectra can be recorded over a range of 4000 – 10 cm^{-1}. However, Raman active normal modes of vibration of organic molecules occur in the range of 4000 – 400 cm^{-1} (Bumbrah & Sharma, 2016). One

important thing to keep in mind when interpreting a Raman spectrum is that a spectral band may not only be assigned based on its band position, but other band parameters such as width and shape may also be of great use. It is also important to note that it might be impossible to assign all of the bands found in a spectrum, so instead of trying to assign every weak band the focus should be on the very intense bands. If the physical state of the analyzed sample is known, it should also be taken into account when interpreting the spectra, as it can strongly influence the spectra by shifting band positions and/or changing the band shape. For identifying an unknown compound most spectroscopic laboratories use computer aided spectrum interpretation e.g. spectral libraries containing a large range of pre-recorded reference spectra (Lotte Boge Lyndgaard, 2013).

Raman spectra consist of two parts, the Raman signal and the noise part. The definition of noise in this context is the part of the spectra containing unwanted information not related to the chemical composition of the sample. The quality of a Raman spectrum if often determined by the signal-to-noise ration (SNR), and it is thus important to increase the signal and reduce the noise as much as possible. The main sources of noise are: *shot noise*, *dark noise*, *fluorescence*, *cosmic rays*, *light sources* and *blackbody radiation*.

Shot noise is usually the dominant source of noise from the CCD. It arises from the random timing and energy of scattered photons. Two consecutive spectral measurements of the same sample will always show some variation in number of photons collected at any pixel. Shot noise level is equal to the square root of the number of detected photons. For a CCD Raman, more detected Raman photons (longer acquisition time) leads to a decrease relative standard deviation and increased SNR in the Raman spectra. In other words, uncertainty decreases with measurement time.

Another type of noise related to the detector is so-called ***dark noise***, which is defined as the rate of spontaneous generated electrons in the detector. The amount of dark noise is strongly dependent on the temperature, and it can thus be reduced by sufficient detector cooling, and by dark subtraction (Lotte Boge Lyndgaard, 2013).

When ***fluorescence*** occurs, the sample is raised into the first excited electronic state by absorption of a photon. The sample rapidly relaxes to the lowest vibrational level of the first excited state and, after a period of time, the sample relaxes back to the ground electronic state by emitting a photon of fluorescence. Fluorescence in a Raman spectrum can be challenging, because the fluorescence signal is often very high compared to the Raman signal, and therefore the Raman signal can be hidden under strong fluorescence. Fluorescence problems are not always predictable and often occur in samples not typically described as fluorescent. That may be due to the fact that even very low concentrations of fluorescent material in a bulk material can produce a fluorescence background that is much stronger than the Raman signal. When considering analyzing a sample with Raman spectroscopy, it is useful to understand what conditions lead to fluorescence background, and how it can be minimized. One way of reducing fluorescence is to use longer excitation wavelength. By applying a longer excitation wavelength

the fraction of common material that can absorb light and create an excited electronic state will decrease. Also illuminating the sample with laser light a period of time before data acquisition begins can sometimes reduce the fluorescence background, due to the destruction of fluorescent material. Finally there are spectral preprocessing methods that can be applied to remove or reduce fluorescence background (Lotte Boge Lyndgaard, 2013).

Cosmic rays appear in the spectrum as very sharp and strong intensity bands. They are common in data of CCD devices. Cosmic ray filtering can usually be applied to eliminate the effect from cosmic rays.

Also sunlight and a range of other ***light sources*** add similar sharp bands in the spectrum. Therefore, Raman measurements should be performed in darkness. And finally, any Raman signal is masked by strong ***blackbody radiation*** which is defined as the radiation all objects emit because their temperature is above the absolute zero (Lotte Boge Lyndgaard, 2013).

Multivariate Data Analysis

Large datasets can be generated using Raman spectroscopy, which creates a challenge in terms of extracting useful information from these complex data. Univariate analysis is considered the easiest, most prevalent and most robust data analysis approach and, in many cases, can provide sufficient information and reliable predictability. The complex and vast amount of data generated using Raman spectroscopy often demands multivariate data analysis approaches (Paudel et al., 2015). With multivariate methods, it is possible to investigate the relations between all variables in a single context. These relationships can be displayed in plots as easy to understand as time traces and pair-wise scatter plots (Guide, 2013). Multivariate analysis is advantageous in aspects like handling of interference, noise reduction, outlier detection, and for exploratory analysis. Most often, very small spectral changes can be detected and several compounds in a sample can be analyzed simultaneously.

Preprocessing

Preprocessing of the spectral data is the most important step before multivariate modeling. In Raman spectroscopy, the spectra are often affected by several factors other than the chemical components of interest, and if the effects from these unwanted factors are not eliminated, they can create challenges in the subsequent qualitative or quantitative analysis. In this dissertation only two preprocessing methods were applied to Raman spectra from tablets with different concentrations of paracetamol.

The Background contribution in a Raman measurement can cause the baseline of the spectrum to vary from zero. Background in the Raman spectrum arise from laser-induced fluorescence, non-laser-induced emission processes (e.g. room light) or Raman scattering of something beside the analyte (solvent, substrate, etc.). Eventually, many Raman spectra contain some unavoidable background, although it may not be strong enough to interfere with quantification. A common method used to remove unwanted background from signal of interest is taking the derivatives of

the measured response. By taking the derivatives a filter is created between high-frequency (sharp and narrow) features like the Raman bands and low-frequency (broad and smooth) features such as the baseline. Savitzky and Golay were interested in smoothing noisy data obtained from chemical spectrum analyzers, and they demonstrated that least squares smoothing reduces noise while maintaining the shape and height of waveform peaks (Schafer, 2011). **Savitzky-Golay** (SG) smoothing permits to replace each data point by some kind of local average of surrounding data points (window of points). Since nearby points measure very nearly the same underlying value, averaging can reduce the level of noise without (much) biasing the value obtained (Press, n.d.). The input to the algorithm is the order of the polynomial and the size of the window. Increasing the window size and decreasing the polynomial order will increase the smoothing. This preprocessing method is also useful to remove the aforementioned shot noise.

A large cause of systematic variation in the Raman spectral measurements arises from total intensity variations i.e. uniform intensity changes throughout the spectrum. The incident intensity will change with laser-intensity output and laser throughput to the sample. Once Raman models are in most cases based on determination of the intensity, they will therefore be influenced by total intensity variations. Usually, a normalization of the entire spectrum is necessary. Normalization is performed by dividing each variable of a spectrum with a constant. **Standard Normal Variate** (SNV) is the preprocessing method that has been applied for all data obtained in this dissertation. Applying SNV, the average and standard deviation of all the data points for that spectrum is calculated. The average value is subtracted from the absorbance for every data point and the result is divided by the standard deviation. Multiplicative effects of scattering can be reduced after SNV transformation (Bi et al., 2016). SVN is applied to every spectrum individually, and not on an entire set of spectra.

Figure 3.8 shows the real effect of the pre-processing SNV and SG applied to the Raman spectra of tablets produced with different concentrations of paracetamol, measured through a white blister package, using the PhAT probe from Kaiser Optical Systems. The first graph corresponds to the raw data, the second graph corresponds to the spectrum processed by SNV to which was applied the SG preprocessing.

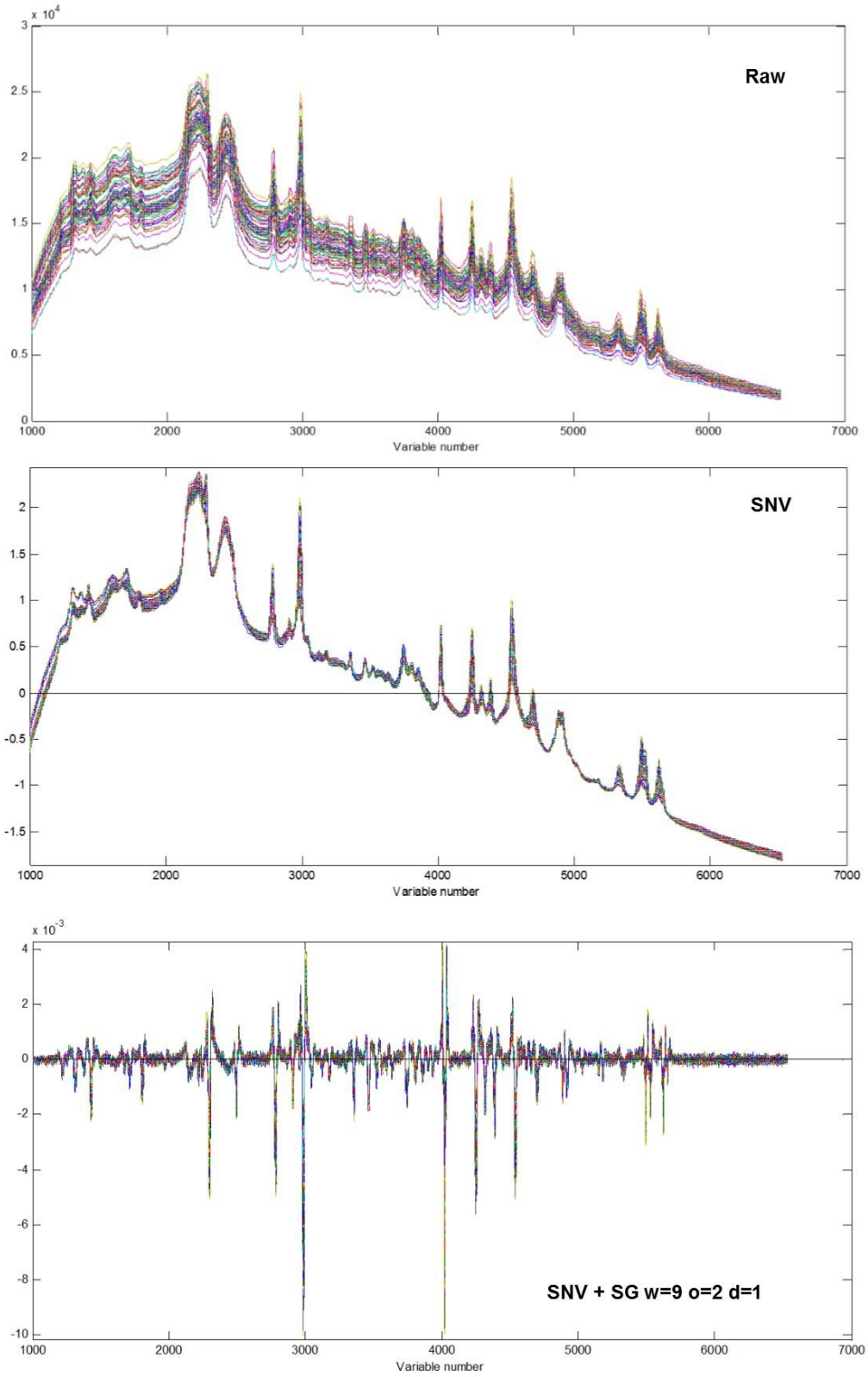

Figure 3.8 The effect of preprocessing of Raman spectra of tablets (with different concentrations of paracetamol) measured through white blister packaging using PhAT probe from Kaiser Optical Systems.[Latentix™]

Qualitative analysis

In qualitative analysis the goal is to find and utilize similarities and differences between samples to group them without prior knowledge. Because of the very well resolved bands found in the spectra, Raman spectroscopy provides a very powerful tool for distinguishing between samples with only minimal differences. However, the variations between spectra can be very small and difficult to identify visually, thus chemometric methods can be useful for exploring spectral data. Approaches used for finding patterns and clusters within a data set are called unsupervised techniques because no prior information needs to be presented to the model prior to analysis (Lotte Boge Lyndgaard, 2013).

Principal component analysis (PCA) is a multivariate technique that analyzes a data table representing observations described by several dependent variables, which are, in general, inter-correlated. Its goal is to extract the important information from the data table and to express this information as a set of new orthogonal variables called principal components (PC). PCA also represents the pattern of similarity of the observations and the variables by displaying them as points in maps (Williams, 2010).

Equation 3 expresses the PCA model. A data matrix, **X**, can be decomposed into a score matrix (**T**) and a loading matrix (**P**), which capture the main variation in data. The PCA also gives residuals (**E**), deviations between the data and the PC model.

$$\mathbf{X}_C = \mathbf{T}\mathbf{P}^T + \mathbf{E} \qquad (3)$$

$\mathbf{X}_c$ is mean centered (subtracting the mean of each variable from the original measurement) or autoscaled (subtracting the mean of each variable from the original measurement and dividing with the standard deviation of each variable). Systematic variations in samples and variables are found in the score matrix (**T**) and loading matrix (**P**), respectively (Lotte Boge Lyndgaard, 2013). By PCA, the main variation in a multidimensional data set is found by creating new linear combinations of the original variables. These linear combinations are called PC. The first PC is the direction through the data that explains the largest variation in the data. The second and subsequent PCs describe the maximum amount of the remaining variation. The number of PCs expresses the number of observed variations in a data i.e. the number of independent phenomena. If original variables in a data set co-vary, the number of PCs is expected to be much smaller than the number of original variables. In case of spectral data such as Raman spectra, where the variables are highly collinear, the data matrix is likely to be decomposed into a considerably lower number of PCs than the number of variables.

The result of a PCA model can be graphically illustrated as a score plot and a loading plot. From the score plot, sample similarities (trends and groupings) can be identified as well as outliers. In Figure 3.9 the score plot of the first PC shows well defined groups of tablets (blends) with different concentrations of paracetamol. The loading plot shows the importance of the original variables

for each PC, e.g. which variables are responsible for the separation of samples or responsible for a particular sample to be an outlier (Amin, Bourget, Vidal, & Ader, 2014).

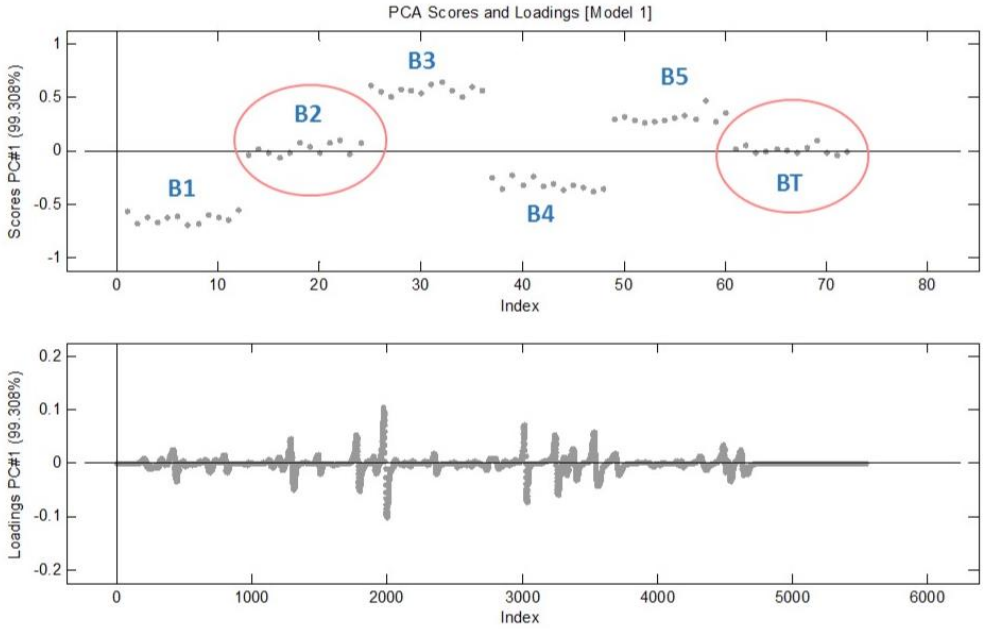

Figure 3.9 PCA score plot and loadings from different blends of tablets measured directly using PhAT probe. Tablets from blend 1 (B1) have the lowest concentration of Paracetamol, blend 2 and blend Test (BT) have the same concentration of paracetamol and blend 3 has the highest concentration.

Quantitative analysis

Under constant experimental conditions, the number of Raman scattered photons is proportional to analyte concentration. This fundamental relationship between intensity and concentration is the basis of using Raman for quantitative analysis. There are different approaches to quantitative analysis of Raman spectra. Sometimes, the intensity of a single band or the ratio of bands can be used for quantification, but in other situations more advanced methods like **Partial Least Squares** (PLS) regression are used. Multivariate calibration models are popular chemometric tools that establish a relation between the spectra in data matrix **X** and the concentrations in data vector (Shehata et al., 2016).

PLS represents the most popular multivariate model for quantitative Raman analysis. PLS modeling has been developed explicitly for a type of situation with numerous, often-correlated input and process variables and several to many result variables (Guide, 2013). In PLS regression, a set of dependent variables **y** or **Y** is modeled from a set of independent variable **X**. The purpose of this is to estimate **Y** from future measurements of **X** only, requiring a representative calibration set with known values of **X** and **Y** (Lotte Boge Lyndgaard, 2013) PLS is well suited for spectroscopic data sets for two reasons: 1) they often contain more variables than samples and 2) many spectral variables co-vary, which means that the number of variables is much higher than the number of independent phenomena in data.

In PLS, the individual variations in **X** and **Y** and their mutual correlation are simultaneously maximized. Explaining PLS is often done by introducing the concept of an outer relation (PCA on **X** and **Y** individually) and an inner relation where the association between **X** and **Y** scores is found by linear regression. However, PLS does not actually work that simple, because when PCA is calculated on the **X** and **Y** separately, their mutual relation will be weak (Lotte Boge Lyndgaard, 2013). PLS is thus a method for constructing predictive models when the factors are many and highly collinear. The emphasis is on predicting the responses and not necessarily on trying to understand the underlying relationship between the variables. In addition to spectrometric calibration, PLS has been applied to monitoring and controlling industrial processes; a large process can easily have hundreds of controllable variables and dozens of outputs (Tobias, n.d.).

3.2 Raman spectroscopy for detecting counterfeits – Studies

For the preparation of this dissertation it was important to acquire some knowledge about previous articles related to detection of counterfeit medicines using Raman spectroscopy. It is also important for the reader to know some of the studies already made in this area in order to comprise the importance of the study carried out for dissertation. Table 3.2 presents several articles sorted by date as well as the correspondent key points that summarize each of them.

The choice for these articles was made by a method of appropriate research to find the required information. The research was carried out in two search engines: **b-on** and **PubMed**, in which were introduced the assignments "Raman spectroscopy", "Detection of counterfeit medicines", "Counterfeit medicines detection", "Raman spectroscopy for detecting Counterfeits" and "Raman applicability spectroscopy ". Other articles were provided by Professor Fransciscus van der Berg.

Table 3.2 Studies regarding the detection of counterfeit medicines using Raman spectroscopy

Article	Key Points	Year	Reference
"New reliable Raman collection system using the wide area illumination (WAI) scheme combined with the synchronous intensity correction standard for the analysis of pharmaceutical tablets"	• WAI Raman scheme • Isobutyric anhydride • Raman intensity • Nanoproxen tablets	2006	(Kim, Chung, Woo, & Kemper, 2006)
"A new non-invasive, quantitative Raman technique for the determination of an active ingredient in pharmaceutical liquids by direct measurement through a plastic bottle"	• Direct measurement • Plastic container • Povidone concentration • WAI Raman scheme	2007	(Kim, Chung, Woo, & Kemper, 2007)

"Detection of counterfeit Viagra® with Raman spectroscopy"	• Counterfeit Viagra • Raman spectroscopy • PCA • Hierarchical Cluster Analysis (HCA)	2008	(de Veij, Deneckere, Vandenabeele, de Kaste, & Moens, 2008)
"Recent advances in the application of transmission Raman spectroscopy to pharmaceutical analysis"	• Transmission Raman spectroscopy • Pharmaceutical analysis • Multivariate techniques	2010	(Buckley & Matousek, 2011)
"Spatially offset Raman spectroscopy (SORS) for the analysis and detection of packaged pharmaceuticals and concealed drugs"	• SORS • Package pharmaceuticals • Drug detection	2011	(Olds et al., 2011)
"Quantification of paracetamol through tablet blister packages by Raman spectroscopy and multivariate curve resolution-alternating least squares"	• Quantification through blister packages • Multivariate Curve Resolution – Alternating Least Squares (MCR-ALS) • Paracetamol in a ternary mixture	2013	(Lotte B. Lyndgaard, Van den Berg, & De Juan, 2013)
"Assessment of the performance of a handheld Raman device for potential use as a screening tool in evaluating medicines quality"	• TruScan handhelded Raman device • Counterfeit medicines • Substandard medicines	2013	(Hajjou, Qin, Bradby, Bempong, & Lukulay, 2013)
"Raman Microscopy for Detecting Counterfeit Drugs — A Study of the Tablets Versus the Packaging"	• Raman microscopy • Study of tablets • Study of packaging • Detecting counterfeits	2014	(Workbench, 2014)
"An iterative approach for compound detection in an unknown pharmaceutical drug product: Application on Raman microscopy"	• Raman microscopy • Unknown pharmaceutical product • Compound detection • Spectral library	2016	(Boiret, Gorretta, Ginot, & Roger, 2016)

In the article "New reliable Raman collection system using the WAI scheme combined with the synchronous intensity correction standard for the analysis of pharmaceutical tablets" a novel and reliable Raman collection system for the non-destructive analysis of pharmaceutical tablets was proposed. The effective and synchronous standard configuration using isobutyric anhydride was harmonized with the WAI scheme to correct the problematic variation of Raman intensity from the change of laser power. To verify the quantitative performance of the proposed scheme, the compositional analysis of active ingredient in naproxen tablet was performed. The average sample composition was successfully represented by using the WAI scheme compared to the conventional scheme with a much smaller illumination area. A PLS model was developed using

an optimized spectral range and the concentrations of naproxen in tablets were accurately predicted. The Raman scheme presented in this study can be used in the field of pharmaceutical analysis and could improve the efficiency of measurements in quality assurance situations. Additionally, the proposed scheme can be utilized for the identification of pharmaceutical raw materials where samples are in diverse forms (Kim et al., 2006).

In the article "A new non-invasive, quantitative Raman technique for the determination of an active ingredient in pharmaceutical liquids by direct measurement through a plastic bottle" direct Raman measurement through a plastic bottle was successfully accomplished for the measurement of an API (povidone) in an eyewash solution using WAI Raman scheme. Simultaneously, isobutyric anhydride was placed in front of the bottles to use for a synchronous external standard configuration helping to correct the problematic variation of Raman intensity from the inherent fluctuation in laser power. The povidone concentration was successfully measured with spectral collection that was performed through a plastic barrier. The result from this study suggests that the WAI scheme exhibits a strong potential for the non-destructive quantitative analysis of pharmaceuticals measured directly in plastic containers. If implemented, this technique could be utilized as a simple and rugged method for quality assurance of final products in a manner consistent with Process analytical technology (PAT) requirements (Kim et al., 2007).

The article "Detection of counterfeit Viagra® with Raman spectroscopy", proposed Raman spectroscopy as a fast and reliable method for the detection of counterfeit Viagra® tablets without the need of sample preparation. In this work, 18 tablets were examined. From visual inspection, 3 tablets could be assigned as counterfeit, whereas Raman spectroscopy was able to detect 9 counterfeit tablets from a test set. By using a combined approach of PCA and HCA, with Raman spectroscopy it was possible to design an automated approach to distinguish between genuine and counterfeit tablets. Raman spectroscopy, combined with PCA, could be used in the future by customs or in the field to identify counterfeit tablets on the spot without involvement of trained chemists (de Veij et al., 2008).

The article "Recent advances in the application of transmission Raman spectroscopy to pharmaceutical analysis" reviews recent advances in TRS and its applications, from the perspective of pharmaceutical analysis. A combination of the benefits of transmission Raman spectroscopy: the ability to yield highly chemical specific information, the ability to probe water containing samples and the ability to obtain quantitative volumetric data from thick and highly turbid samples, unlocks a range of new applications in pharmaceutical settings including quantitative bulk analysis of intact pharmaceutical tablets and capsules in quality and process control. These include rapid volumetric quantification of API/excipients and of polymorphs within intact tablets and capsules or powders. Perhaps most importantly, the technique is experimentally straightforward and can be combined with the existing multivariate techniques (Buckley & Matousek, 2011).

The article “Spatially offset Raman spectroscopy (SORS) for the analysis and detection of packaged pharmaceuticals and concealed drugs” demonstrates the SORS technique in several scenarios that are relevant to customs screening, postal screening, drug detection and forensics applications. The examples include analysis of a multi-layered postal package to identify a concealed substance; identification of an antibiotic capsule inside its plastic blister pack; analysis of an envelope containing a powder; and identification of a drug dissolved in a clear solvent, contained in a nontransparent plastic bottle. The results presented in this article show the utility of SORS for detecting various types of drugs and pharmaceuticals in a range of non-transparent containers. The key advantages of SORS are its noninvasive and non-contact nature, relatively short analysis times, the capability of penetrating opaque (diffusely scattering) containers, and its ability to obtain chemically specific data to identify concealed substances. The authors say that SORS will also contribute to the technology used to scrutinize and inspect the production of tablets, capsules and other pharmaceutical products, and may help prevent the dangerous and increasing trend of counterfeits (Olds et al., 2011).

In the article “Quantification of paracetamol through tablet blister packages by Raman spectroscopy and multivariate curve resolution-alternating least squares” is demonstrated how paracetamol as part of ternary mixtures can be quantified inside blister packages using the combination of Raman spectroscopy and MCR-ALS. This was the first study to quantify paracetamol through blister packaging. An MCR-ALS model with correlation constraint could predict paracetamol concentrations in a test set with an error of 0.8% w/w in the concentration range 0–20% w/w. In addition, the authors propose a modification of the correlation constraint in MCR-ALS as a way to improve quantification of a compound in a dataset with varying interferences. The interference in this study was caused by variations in blister material, and it gave rise to a difference in the relationship between concentration and spectral response. These variations interfere with the Raman signal and therefore make quantification challenging with conventional regression methods. Further optimization by adding a rescaling step of the concentration values was also implemented in ALS optimization to account for the matrix effect caused by the presence of the blister. Using this approach paracetamol concentration could be predicted through blister package despite variation in packing material. Models with prediction errors of 1.3, 1.4 and 1.9% w/w were obtained for samples measured through three distinct blister packages. The novelty in the modification allows working with a multiset structure, making local regression models that can handle both signal interferences and matrix effects (e.g. packing material, temperature, instrumental variations) (Lotte B. Lyndgaard et al., 2013).

The article “Assessment of the performance of a handheld Raman device for potential use as a screening tool in evaluating medicines quality” reports on the TruScan® handheld Raman device’s ability to discriminate between a specific product and similar products from different manufacturers, unrelated medicines, and medicines with different strengths. This investigation evaluated its ability to differentiate between similar drug products of similar or different strengths,

focusing on the specificity and precision of the testing. The results of our investigation suggest that the device could be used to detect some counterfeit medicines. The instrument is very likely to detect counterfeit medicines that lack API or have the wrong API; however, some factors should be taken into consideration before using the device in testing for counterfeit medicines. This study demonstrates that a TruScan® unit may not be adequate for detecting substandard finished pharmaceutical products. It failed to distinguish between different strengths of the same medicines tested in our laboratory. Similar to other screening tools, some quality attributes such as dissolution and uniformity of content, cannot be assessed with this device (Hajjou et al., 2013).

The article “Raman Microscopy for Detecting Counterfeit Drugs — A Study of the Tablets Versus the Packaging” presents results of tablet matching measurements and characterization of packaging using Raman microscopy. Raman mapping of tablets enables not only the identification of components, but also the mapping pattern can enable matching manufacturing sites of different tablets. The study of packaging materials can provide an identification of coating and a depth-profile measurement of thickness. Raman microscopy can also provide spectra of dyes and pigments, even if they are printed below a surface film (Workbench, 2014).

In the article “An iterative approach for compound detection in an unknown pharmaceutical drug product: Application on Raman microscopy” an iterative approach was proposed to identify the compounds in a pharmaceutical drug product, assuming that the chemical composition of the product was not known by the analyst and that a low dose compound could be present in the studied medicine. The proposed approach uses a spectral library, spectral distances and orthogonal projections to iteratively detect pure compounds of a tablet. Since the proposed method is not based on variance decomposition, it should be well adapted for a drug product which contains a low dose product, interpreted as a compound located in few pixels and with low spectral contributions. By using the pure identified spectra, multivariate curve resolution-alternating least squares was applied on the whole spectral matrix. Results provided the distribution maps of each compound in the tablet. Distributions of the two crystalline forms of active and the five excipients were in accordance with the theoretical formulation. This approach could be particularly interesting for analyst in either the case of identification of pure compound in an unknown product such as a counterfeit product or during the stability study of a pharmaceutical drug product (tablets, powders or extrudates) to study the degradation of active and the modification of crystalline forms (Boiret et al., 2016).

Chapter 4 - Materials and Methods

The aim of this dissertation is to explore the detection of counterfeit medicines using Raman spectroscopy techniques for non-invasive and non-destructive analysis, in view of its applicability *in the field* (hence, in locations such as borders or places where inspection and distribution of medicines is carried out) and not in laboratories with good resources as well as specialized personnel. This investigation is related to the previously mentioned article "Quantification of paracetamol tablet through the blister packages by Raman spectroscopy and multivariate curve resolution - alternating least squares" in order to thoroughly explore the detection of different concentrations of paracetamol through blister packages.

This chapter begins with a description of an investigation into differences within packaging material. Since the aim of this dissertation is to study de detection of medicines through package it is relevant to investigate if there are differences within the package material and how these differences affect the spectrums of the tablets under analysis. Aluminum blisters were also investigated to find some signal through this material.

For experiencing the detection of different concentrations of paracetamol through blister packages calibration tablets were made. The blisters used were white blisters of "*Ben-u-ron* tablets" from *Bene Farmacêutica*. Furthermore, in this investigation three different probes on two different instruments were used for the measurements: the PhAT probe and MKII probe attached to Raman Workstation microscope, and a green laser (cheaper but less accurate) to compare results.

In this chapter the description of the production process of tablets is presented, followed by the description of the method of analysis of the tablets through the white blister pack with each probe. After the measurements, spectra were analyzed using *Latentix™ software*. *Latentix™* was developed with the intention to make low cost professional software for multivariate analysis so that Chemometrics and multivariate models could be available to everybody in all technological and scientific disciplines, where complex data tables need exploration or efficient data analysis. This idea connects with the idea inherent to this dissertation which is the investigation of a method of analyzing medicines that is easy to use for people without a very high level of training.

4.1 Blister Packages

● It is important to investigate if there are differences within the package material. For this purpose, two empty transparent plastic bottles were analyzed using the PhAT probe. For each of these bottles, ten spectrums of different sites were acquired in order to analyze the differences between the spectrums and verify if there are significant changes between sites in the same bottle. The measurements were made using an exposure time of 10 seconds and the accumulation number of 2, applying 400mW of laser power.

● It is important to investigate how these differences within the plastic material affect the resulting spectrums. Regarding the differences observed in the plastic material packages, sixteen solutions of 40 ml were prepared using water, ethanol and sugar in different concentrations, and introduced into bottles equal to the bottles analyzed previously (Figure 4.). Three measurements of different

sites of the bottles were acquired using PhAT probe, for each one of the sixteen bottles. The measurements were made using an exposure time of 10 seconds and the accumulation number of 2, applying 400mW of laser power.

● The same measurements in package material were repeated with quartz bottles (Figure 4.1). Three quartz bottles, one with water, another one with sugar and another with ethanol, were analyzed in 5 different positions. This material is more expensive but does not affect the spectra obtained as the measurements through plastic bottles. This is useful to obtain exact spectra of each ingredient of the solutions, in order to identify them is the spectrums acquired through plastic bottles. The measurements were made using an exposure time of 10 seconds and the accumulation number of 2, applying 400mW of laser power.

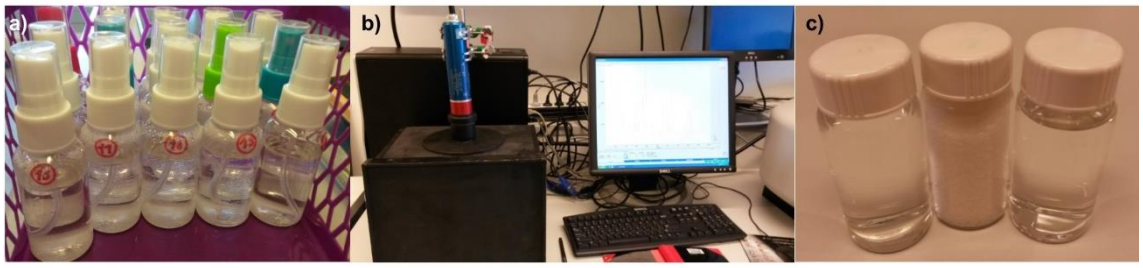

Figure 4.1 Investigation of differences within package material. a) Plastic bottles with a solution of water, sugar and ethanol; b) PhAT system; c) Quarts Bottles with water, sugar and ethanol

● In order to verify the possibility of analyzing medicines through different material an extra investigation was included. Besides analyzing paracetamol tablets through white blisters packages, a short verification of spectra from tablets of Pamol measured through transparent material and aluminum material was made. These measurements were also performed using the PhAT probe and using an exposure time of 10 seconds and the accumulation number of 2, applying 400mW of laser power.

Figure 4.2 Tablets of Pamol measured through transparent material and aluminum material

4.2 Production of tablets

To investigate the detection of different concentrations of paracetamol in tablets using Raman spectroscopy, it is essential to know the exact concentrations of paracetamol and the other excipients, in order to be able to properly analyze the spectra obtained and to verify the efficacy of these detection methods through blister package. Therefore, tablets were produced in the laboratory for this purpose following a previously prepared plan for concentrations of paracetamol. This plan is shown in Figure 4.3.

Six blends of 12 tablets each were produced, and it was decided that all tablets had a total weight of 500mg. From Blend 1 to Blend 5 all have different concentrations of paracetamol ranging between 50mg and 150mg. This plan is based on the production of three blends (1, 2, and 3) with the following concentrations of paracetamol: 50mg, 100mg and 150mg respectively. From these blends two other appear which result from the mixture of Blend 1 and Blend 2 with a ratio of 50:50 (Blend 4) and the mixture of Blend 2 and Blend 3 in a ratio of 50:50 (Blend 5), resulting in two blends giving rise to tablets with 75mg and 125mg of paracetamol, respectively. The same procedure was applied for the production of a blend test (Blend T) with a concentration theoretically equal to Blend 2, if the mixing process was properly executed.

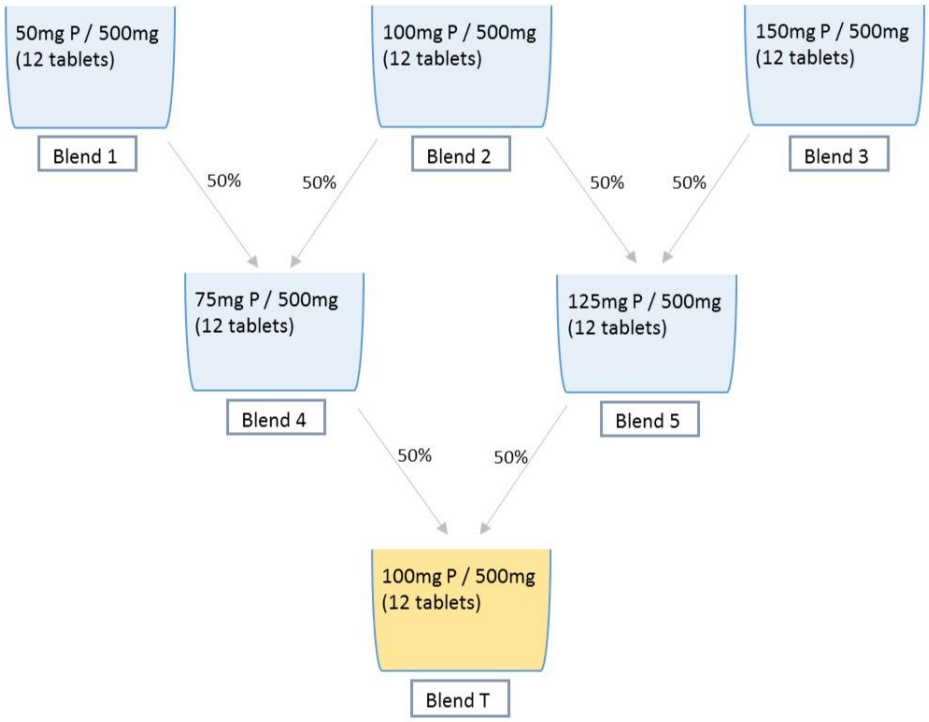

Figure 4.3 Plan for producing blends with different concentrations of paracetamol used to produce tablets for further analysis.

The tablets used in this study were produced from 4 component mixtures: 4-Acetamidophenol, 98% (referred to as paracetamol throughout this dissertation), Potato Starch, α-Lactose and Baby Powder (designated as talc in this dissertation).

Paracetamol is the active ingredient. Potato starch is an excipient often used in the pharmaceutical industry as binder and as a disintegrant. Binders must add sufficient cohesion to the powders and disintegrants enable tablets to break down into smaller fragments (dissolve) so that the medicine can be released for absorption. Starches also absorb water rapidly, allowing tablets to disintegrate appropriately ("Drugs.com. Know more. Be sure.," n.d.). Lactose mono-hydrated is commonly used as a diluent in solid dosage forms (tablets and powders) ("FARMACOPEIA PORTUGUESA VIII," n.d.). It is added to the powders to allow to obtain tablets with appropriate volumes. To provide flow and compression properties talc is commonly used as a tablet diluent and as a lubricant. Lubricants prevent sticking of the tablets to the tablet punches during the compression phase of the tablet manufacturing process (Rutesh H. Dave, PhD, Assistant Professor, Division of Pharmaceutical Sciences, 2008). It is important to note that these are simple tablets with only 4 components that aim to simulate real medicines with different concentrations of paracetamol to be recognized.

Table 4.1Table 4. shows the amounts (by weight) of the components of a 500mg tablet for each of the blends from 1 to T. After the planning for paracetamol concentrations of each blend (Figure 4.3), it was decided that the amount of talc would be 10mg for all tablets and the amount of lactose and starch would be equal to each other. In total 72 tablets were produced and analyzed.

Table 4.1 Amounts of each excipient for a tablet with 500 mg of total weight

Blend	Paracetamol (mg)	Starch (mg)	Lactose (mg)	Talc (mg)	Total (mg)
1	50	220	220	10	500
2	100	195	195	10	500
3	150	170	170	10	500
4	75	207.5	207.5	10	500
5	125	182.5	182.5	10	500
T	100	195	195	10	500

The tablet production method was a simple method in which existing equipment in the laboratory was utilized, which is not specifically suitable for the production of "true" paracetamol tablets. Sterilization techniques were also not applied since these procedures are not relevant to this investigation. The production method consisted on three distinct steps: weighing, mixing and compression.

Regarding the weighing of the right amount of excipients for each blend, some calculations were made. These calculations were made for excess since some part of the material adhering to containers leads to a lack of product for compression. Table 4.2 shows the values of the total required amounts of each of the excipients as well as the quantities necessary for each of the blends. The values presented in this table were calculated from the values presented in Table 4.2. Taking as an example the amount of paracetamol required for blend 1: the 0.5g of

paracetamol (required for one tablet) multiplied by 12 (12 tablets necessary for each blend), results in 0.6g; this value multiplied by 4 giving rise to the 2.4 g of paracetamol presented in Table 4.2. In this way there is a sufficient amount of excipients for producing 12 tablets from blend 1 and also for follow-up blends 4 and T.

Table 4.2 The necessary amounts of each excipient

Blend	Paracetamol (g)	Starch (g)	Lactose (g)	Talc (g)	Totals (g)
1	2.4	10.56	10.56	0.48	24
2	4.8	9.36	9.36	0.48	24
3	7.2	8.16	8.16	0.48	24
4	3.6	9.96	9.96	0.48	24
5	6	8.76	8.76	0.48	24
T	4.8	9.36	9.36	0.48	24
Totals (g)	28.8	56.16	56.16	2.88	

Each Blend (from 1 to T) was mixed manually using glass marbles (Figure 4.4) into sterile vials for an improved homogeneity of the mixture. After the blends were finished, 72 tablets of 500mg each were compressed by direct compression, using a Perkin-Elmer compressor and applying a force of approximately 30 000 Newton. During this process a vacuum system was used to facilitate uniformity of tablets with regard to its thickness. This system helped spreading the powder across the die before compression.

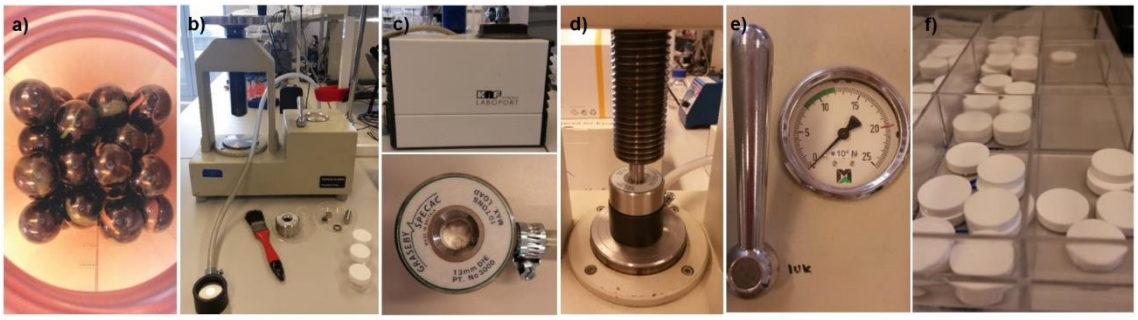

Figure 4.4 Mixing and compression steps from production of paracetamol tablets. a) Sterile vials with glass marbles for better homogeneity; b) Perkin-Elmer compressor; c) Vacuum system; d) Process of direct compression; e) Pressure display and f) Final paracetamol tablets.

4.3 PhAT Probe

The first measurements were performed using the PhAT probe from *Kaiser Optical Systems*. With a laser wavelength of 785nm and spectral coverage of 175-1875cm^{-1}, the *PhAT* probe represents the *in situ* approach to Raman solids sampling. The problem of under-sampling in solids is solved using the *PhAT* approach, which provides reproducible, representative, focus-free, quantitative

Raman measurements. Its 6 mm large-area laser spot size allows a significant volume of the sample to be interrogated in a single measurement. The *PhAT* approach eliminates the need for multiple measurement points, the need to move samples, and eliminates focusing, thus speeding up analysis time (“Kaiser, optical systems, inc.,” n.d.).

The PhAT System (Figure 4.5a) addresses the sensitivity needs of Raman spectroscopy by processing and detecting Raman photons very efficiently. The spectrograph section of the PhAT System provides complete spectral coverage, good spectral resolution, low scatter, and high throughput. Unlike traditional Raman instruments, the PhAT System is easy for a non-specialist to use. Good engineering allows the user to ignore the product's state-of-the-art technology and focus on the application. The fiber optic probe brings point-and-shoot intuitive simplicity to Raman sample alignment. This System collects data quickly, allowing complete, full-range spectra collected in a few seconds. The PhAT System is not tied to a scientific laboratory. It delivers the same high performance on a plant floor or office desktop that it does in a laser laboratory. It is a small, single-package unit that could be carried by an individual or by a small cart. It works well in any orientation, including upside down. It is rugged enough to maintain near-optimal calibration when moved. The PhAT System is bringing the power of Raman spectroscopy out of the laboratory and into the real world (Kaiser Optical Systems, 2004).

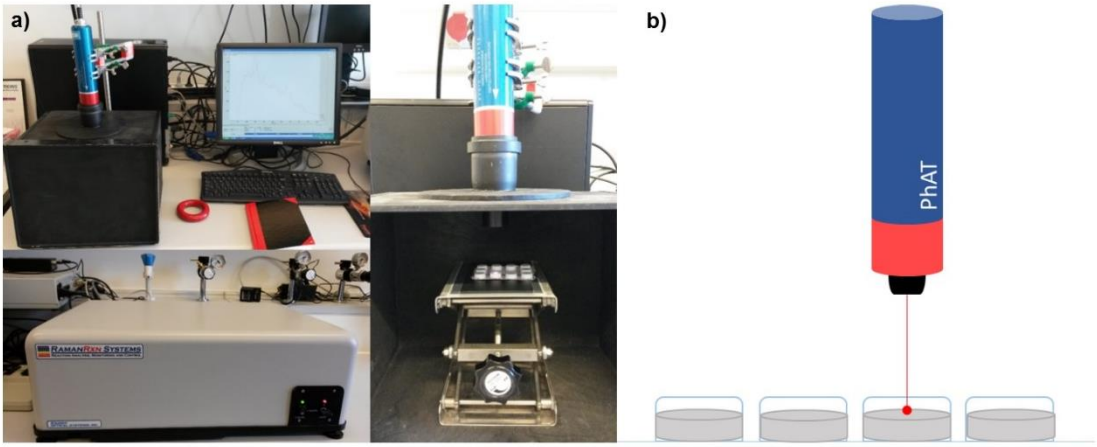

Figure 4.5 Phat system (a)) including the PhAT probe and RamanRxN System and measuring scheme (b))

Firstly, a direct measurement of all the tablets was carried out using PhAT probe. In addition, it was also carried out a measurement of an empty white blister. Thereafter, the twelve tablets from each of the six blends (from 1 to T) were measured according to a specific measurement scheme. The measurement scheme of the tablets through the blister package is shown in Figure 4.5b. As can be seen, the normal position of a blister pack includes an air space between the tablets and the blister. This space does not constitute an obstacle for measurements with PhAT probe. The measurements were made using an exposure time of 20 seconds and the accumulation number of 1, applying 400mW of laser power. At the beginning of each set of measurements a dark was

collected and every time a measurement was carried out, the system collected a cosmic ray correction spectrum automatically.

4.4 MKII Probe

After the measurements with PhAT probe, the same measurements were carried out using Raman Workstation, with the MKII probe from *Kaiser Optical Systems* as a stable interface for Raman Microprobe. The Raman Workstation (Figure 4.6) is designed to provide measurements in minutes. The same measurements can take hours or days on a traditional Raman microscope. The Mk II probe head allows a wide range of optics to be attached. Non-contact optics with working distances of 0.1 to 17 inches can be used to measure through windows, bottles, or simply at a convenient distance from a sample. In this case, the measurements were made through a white blister package using a laser wavelength of 785nm (“Kaiser Optical Systems,” n.d.).

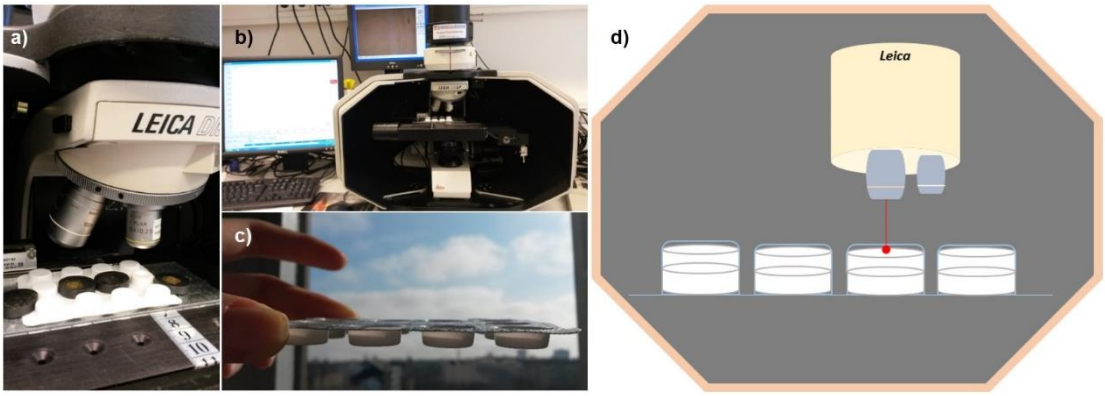

Figure 4.6 Raman Workstation and measuring scheme. a) Objective lens of microscope and blister package with tablets being analyzed; b) Raman Workstation; c) position scheme to be simulated; d) measuring scheme.

The twelve tablets from each blend (from 1 to T) were analyzed using the system presented in Figure 4.6. This system of analysis does not allow the detection of the paracetamol tablets with an air space between tablet and blister package like the PhAT probe does. However, this system enables its use upside down, if mounted in a different configuration than shown in Figure 4.6. Thus, it was necessary to simulate the upside down position (Figure 4.6c) to take the measurements. For this, the measuring scheme presented in Figure 4.6d was utilized, where two tablets were introduced into the same blister cavity of creating height so that the tablet being analyzed could be close to the blister surface.

The MKII probe does not provide a large sampling area as PhAT probe does; therefore, instead of performing only one measurement per tablet, three measurements of random points were performed for a better statistical sampling, closer to the sampling of PhAT probe, so that in the end, the results could be fairly compared. The measurements were made through a 10x/0.25 objective lens using an exposure time of 5 seconds and an accumulation number of 1 with 400mW

of laser power. Every time a measurement was carried out, the system collected a cosmic ray correction spectrum automatically.

4.5 Green laser

Since the theme of this dissertation is counterfeit medicines spreading all over the world, and since this is an even bigger problem in developing countries where the financial possibilities for obtaining drug analysis material are reduced, a green laser instrument, cheaper but less accurate than the previous ones, was also considered for investigating the possibility of detecting different concentrations of paracetamol, and therefore the detection of counterfeit medicines.

The green laser, operating at the 532nm wavelength, suits as an inexpensive light source for Raman spectroscopy (Figure 4.7). Its optical output power is 150 mW.

Just like the MKII probe, this system of analysis does not allow the detection of the paracetamol tablets with an air space between tablet and blister package. Therefore, the measuring scheme was similar to the one utilized for MKII probe, simulating the inverted position of blister package with two tablets inside. Regarding the sampling area of the green laser, this seems to be even greater than the sampling area of PhAT probe due to the projection of the green light emitted by the green laser; however there is no certainty of this. For each of the tablets only one measurement was carried out, just like for measurements with the PhAT probe. The measurements were made using an exposure time of 20 seconds and the accumulation number of 1.

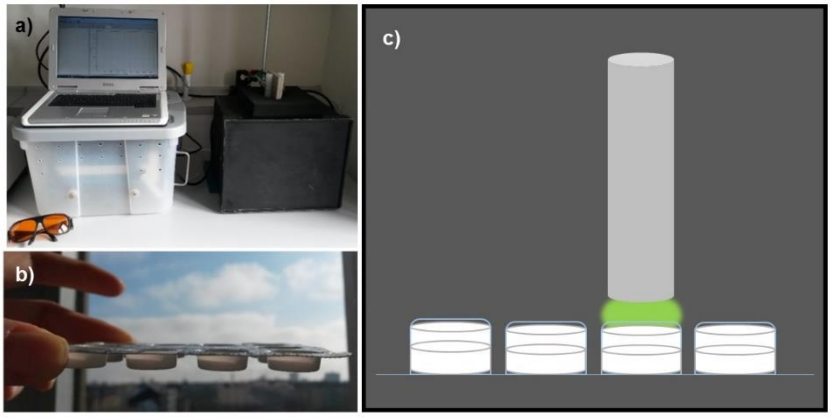

Figure 4.7 Green laser system. a) Green laser equipment setup for the measurements of tablets through white blister package; b) position scheme to be simulated; c) measuring scheme.

Chapter 5 - Results and Discussion

This chapter presents the results concerning the investigations carried out as described in the previous chapter of this dissertation. The results correspond to some initial investigations regarding blister package and the measurements of the 72 tablets from different blends produced for this research, which were analyzed using the PhAT probe, the MKII probe and the Green laser through a white blister package. In this chapter, the aim is to verify the reliability of the detection of different concentrations of paracetamol present in the tablets measured through the package.

5.1 Blister package

● As can be observable in Figure 5.1, clear differences exist between different spots in the same bottle. This happened not only for one bottle of the two investigated bottles, which means that other bottles also have differences within the material. These differences vary in a considerable range of intensity. These observable differences may e.g. be due to variations on the density of the material as can be seen in in Figure 5.2.

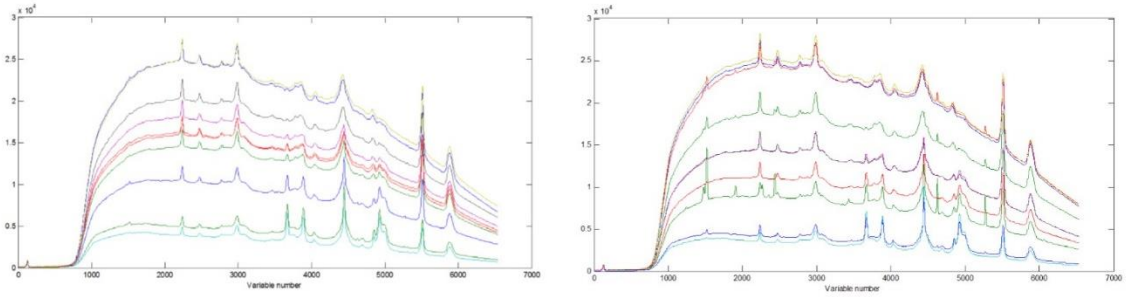

Figure 5.1 Spectrums of two empty plastic bottles in ten random sites. Each plot corresponds to one bottle. [PhAT probe] [Latentix™]

Figure 5.2 Differences in density of plastic observable through a polarization filter in front of polarized light

● Thereafter it is relevant to investigate how these differences within the plastic material affect the resulting spectrums and how they can be overcome. Sixteen solutions of 40 ml were prepared using water, ethanol and sugar in different concentrations, and introduced into bottles equal to the bottles analyzed previously. Figure 5.3 also presents considerable and clearly differences in spectrums of different spots in the same plastic bottle, but this one has a solution of water, ethanol and sugar inside. This helps to observe that variations presented in Figure 5.4a (of all the three measurements for each one of the sixteen bottles) are not only due to variations in concentration of the solutions.

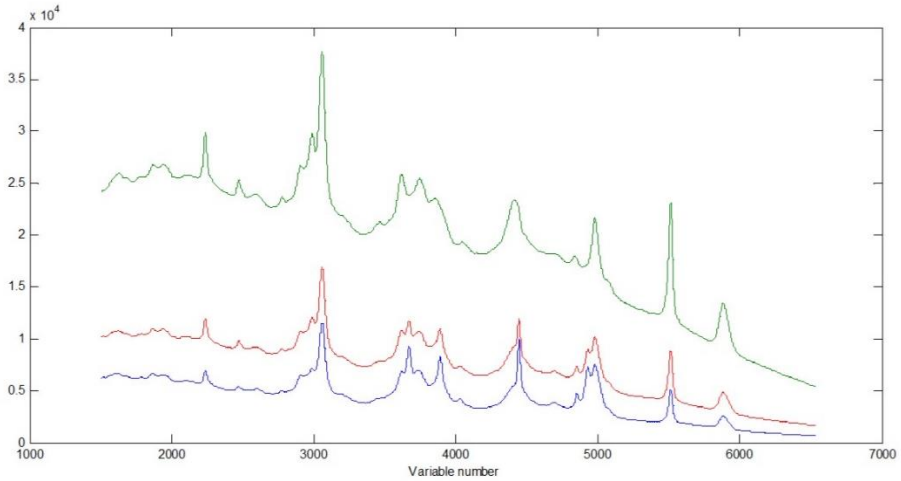

Figure 5.3 Differences between spectrums of three different spots in the same plastic bottle with a solution of ethanol, sugar and water. [PhAT probe][Latentix™]

In Figure 5.4 it is observable that when the raw data from the measurements of the sixteen bottles was preprocessed with SNV, SG and Mean Centered this gave rise to a much clearer spectrum without considerable variations related to plastic differences.

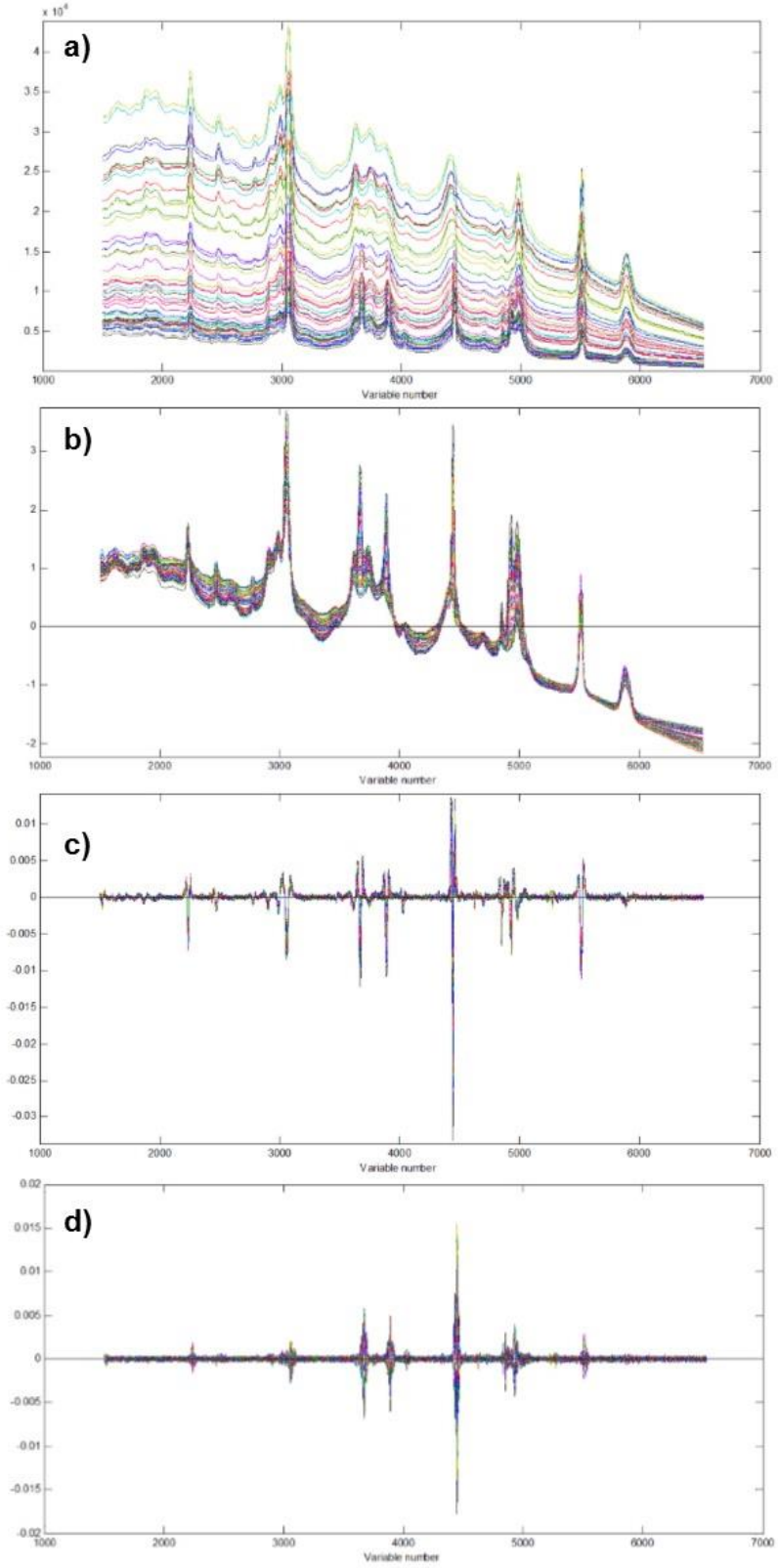

Figure 5.4 Differences between spectrums of three different spots in sixteen plastic bottles with a solution of ethanol, sugar and water. a) Raw data; b) Data preprocessed with SNV; c) Data preprocessed with SNV and SG; d) Data preprocessed with SNV, SG and Mean center [PhAT probe] [Latentix™]

● As can be seen in Figure 5.5 quartz bottles give rise to minimum variations between measurements; peaks of ethanol and sugar are very well defined without almost any interference of the material.

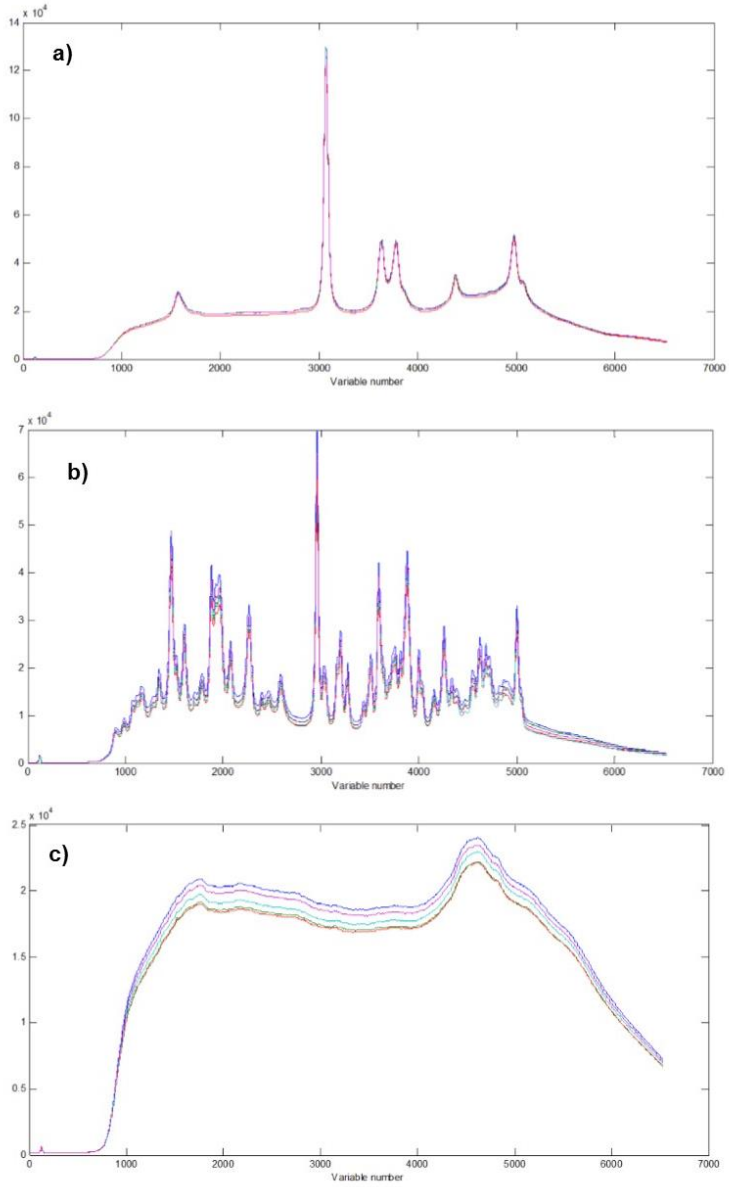

Figure 5.5 Measurements in package material made to quartz bottle. a) Ethanol through quartz bottle; b) sugar through quartz bottle; c) water through quartz bottle. [PhAT probe] [Latentix^{TM}]

● As can be observed in Figure 5.6, Pamol tablets were measured directly as well as through transparent blisters. This results show that no significant interferences can be found in spectrums of tablets through transparent material, which means that tablets in transparent blisters – or at least this specific transparent polymer - can be easily inspected using Raman spectroscopy.

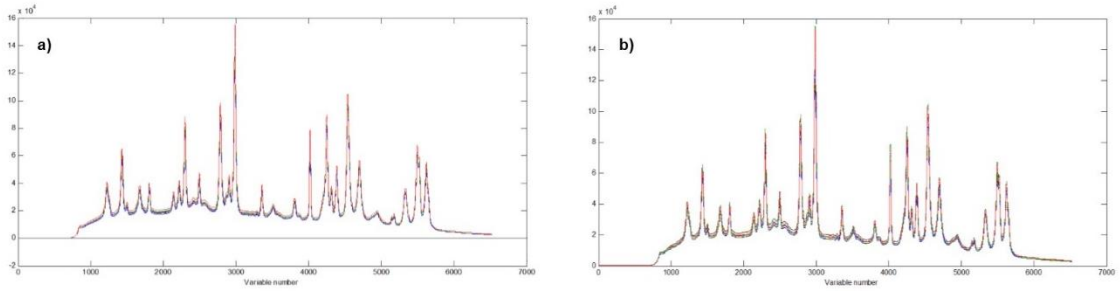

Figure 5.6 Spectrums of Pamol tablets measured directly (a)) and Pamol tablets measured thorugh transparent material (b)) [PhAT probe] [Latentix™]

As can be observed in Figure 5.7, Pamol tablets were measured through aluminum material. The results show that aluminum does not allow Pamol tablet signals to be captured.

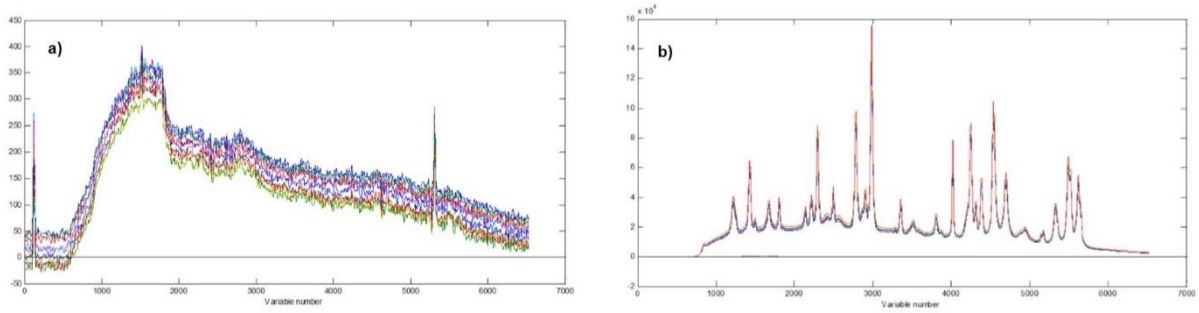

Figure 5.7 a) Pamol tablets measured thorugh aluminium material; b) Pamol tablets measured directly and Pamol tablets measured thorugh aluminium material toghether in the same plot [PhAT probe] [Latentix™]

5.2 PhAT probe

Utilizing the measuring scheme presented in de previous chapter, the twelve tablets from Blend 1 were analyzed directly using the PhAT probe, as well as the empty white blister package and the blister with the tablets inside, in order to observe the differences between these spectra. These differences are shown in Figure 5.8 and Figure 5.9. By comparing the spectra from the empty blister – Figure 5.8b - with the spectrums of the blister with tablets inside – Figure 5.8a – clearly visible peaks allow to identify tablets inside de blister pack. These peaks are clearly recognized in the spectrums of the tablets measured directly, observed in Figure 5.9. It is important to note that these spectrums are not preprocessed.

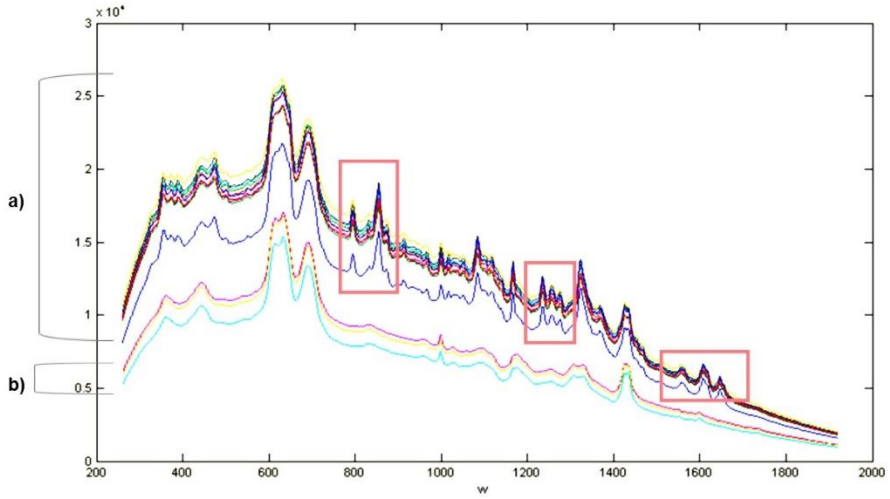

Figure 5.8 Comparison between data from empty blisters and blisters with tablets inside. a) Tablets from Blend 1 through the blister; b) Empty white blister. [Latentix™]

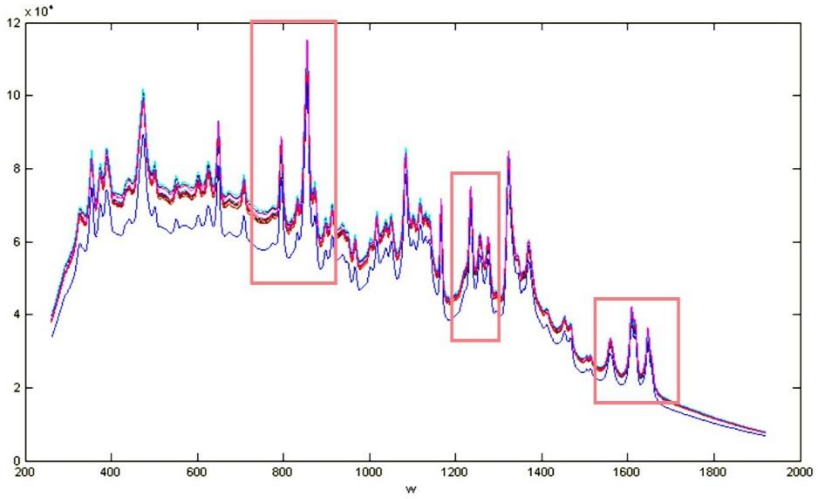

Figure 5.9 Twelve tablets from Blend 1 measured directly (without blister package). The three picks contoured can be easily detected even through the blister package. [Latentix™]

All the spectra used in this dissertation from here on were preprocessed using SNV, SG and Mean Center in this exact order, including the spectrums obtained using other probes. The use of these three simple pre-processing methods in all spectra facilitates the treatment of spectra by people not specialized in multivariate data analysis. The Figure 5.10 presents the resulting spectrum of paracetamol tablets measured directly, after the application of preprocessing methods.

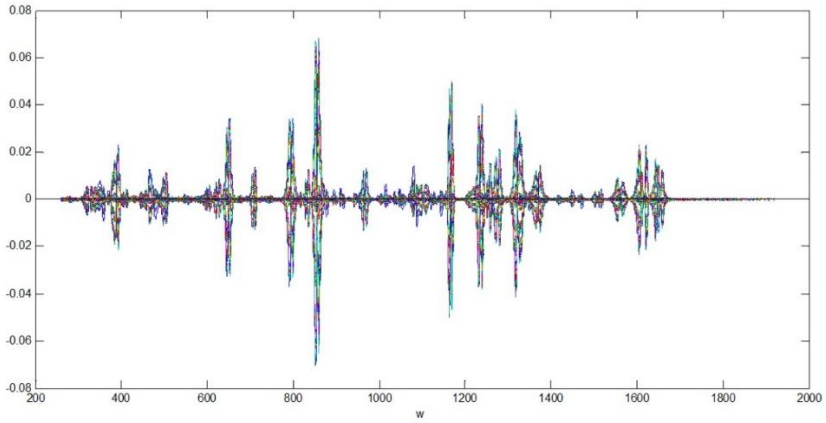

Figure 5.10 Spectrum of Tablets from Blend 1 to Blend T, measured directly. This spectrum is treated with preprocessing methods. [Latentix™]

After treating the spectra, a PCA was performed to this data. Figure 5.11 shows the projection of scores for PC1 and PC2, according to the concentration of paracetamol. Here it is observable that different blends are well defined in this plot, which means that different concentrations of paracetamol in the tablets, are easily detected using Raman spectroscopy. This is not a relevant point for this investigation but it is important to make sure that the blends are well made, therefore different blends should be detected. In this figure it is observable there are two blends with the same concentration of paracetamol (in blue) which refers to Blend 2 and Blend T. Blend T was made with the purpose of confirm de mixing process, which means that the mixing process was good.

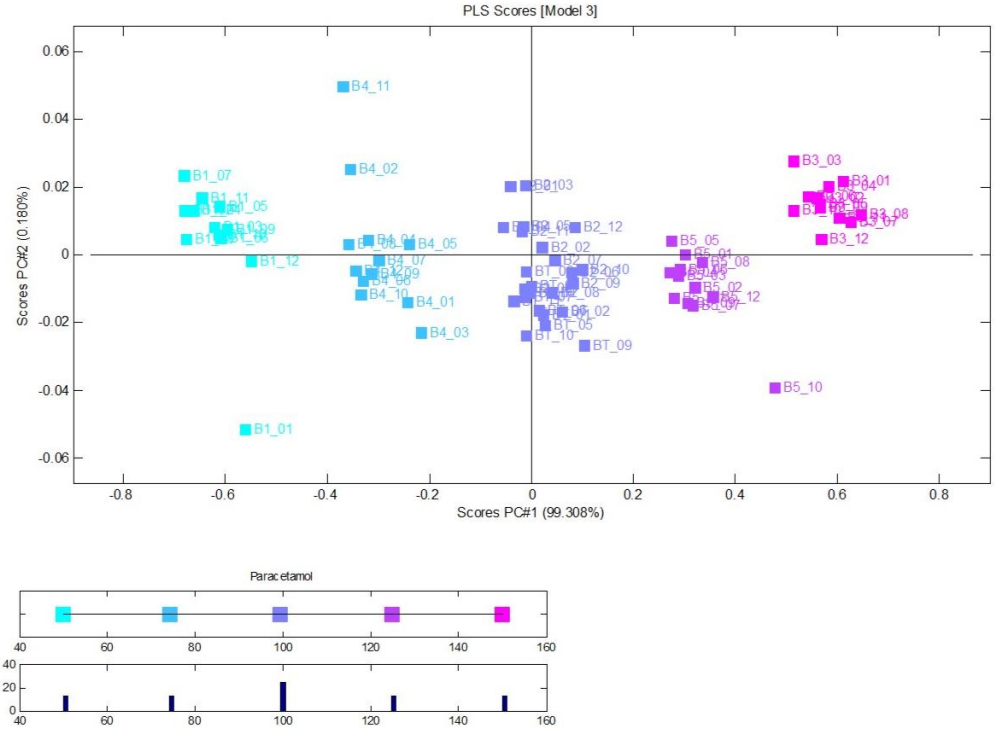

Figure 5.11 Paracetamol Tablets Analysis – Projection of scores for 1 and 2 PC, according to the concentration of paracetamol. [PhAT probe] [Latentix™]

The detection of different blends through white blister, using the PhAT probe was performed. Resulting spectra from these measurements as well as the effect of the different preprocessing methods can be found in the Appendix I. After PCA of these data, a projection of scores for PC1 and PC2, according to the concentration of paracetamol, was acquired (Figure 5.12). In this scores, different blends are not so well defined as in Figure 5.11 (direct measurements) but still, they are perfectly recognizable which means that tablets with different concentrations of paracetamol can be distinguished through a white blister package using PhAT probe.

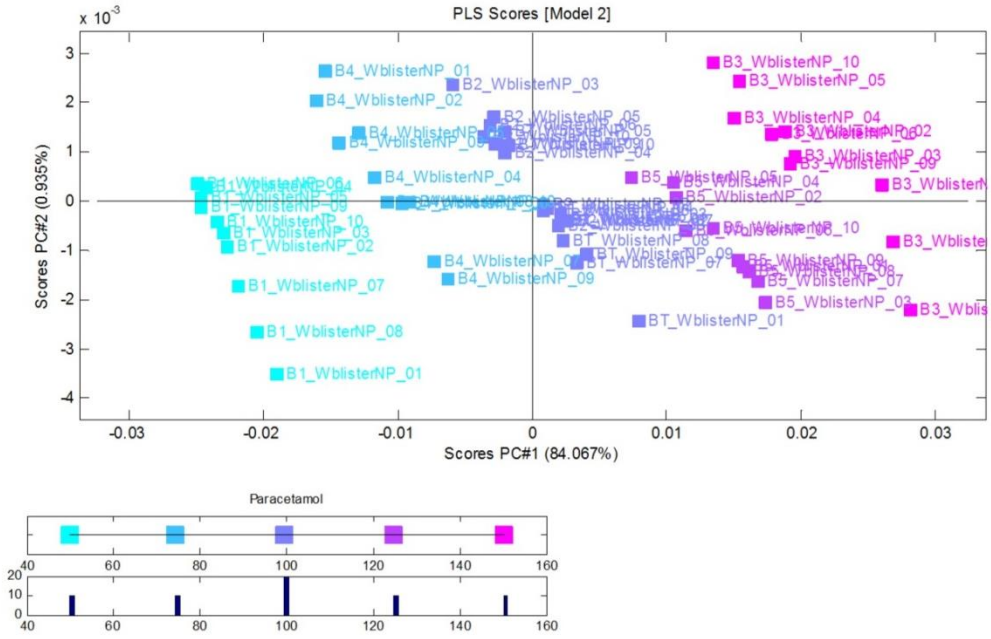

Figure 5.12 Detection of different blends through white blister package – Projection of scores for 1 and 2 PC, according to the concentration of paracetamol. [PhAT probe] [Latentix™]

The big purpose of this investigation is to apply Raman spectroscopy for detecting counterfeit medicines. Thus, PLS models were developed for evaluating the effectiveness of this detection. Two cross-validation methods were tested: "*take 1 blend out*" and "*take 1 tablet out*". In the first mentioned validation method ("*take 1 blend out*") the system uses data from one blend for testing its own prediction, so the data from other blends are used for predicting the blend that is set aside, and so on for each blend. In other words, the blend that is "out" is used for validation and the other blends are used for calibration. As a result Figure 5.13 shows a PLS model statistics for this validation method. From this plot, the ideal number of PC for describing the system can be determined. As can be seen in this plot, the best number of PC is the number that corresponds to the highest value of the r^2 for validation. This value indicates how much the model can explain the values observed, in percentage. Therefore, in this case, 3 PCs is the ideal number of PCs for this validation method, with an $r^2 = 0.9764$. This is a high r^2 value which indicates that this predicting model is robust. As observed in Figure 5.13 calibration values of r^2 are higher than those for validation. This is because the predicting model is performed using the calibration values (or predicting values).

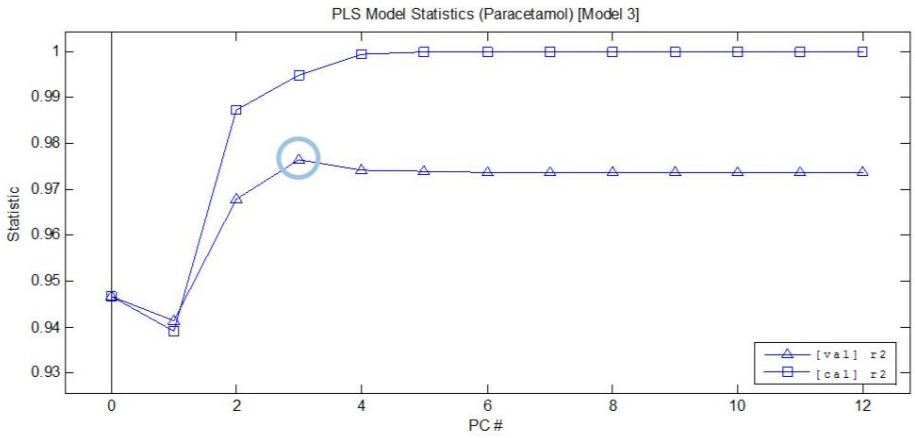

Figure 5.13 PLS model statistics for the validation method "take 1 blend out". [PhAT probe] [Latentix™]

In the second mentioned validation method ("*take 1 tablet out*") the system uses data from on tablet at a time for testing its own prediction and the data from the other tablets is used for predicting the tablet that is missing. That is, the tablet that is "*out*" is used for validation and the other tablets are used for calibration. As a result Figure 5.14 shows a PLS model statistics for this validation method. In this case, 3 PC is also the ideal number of PC for this validation method, with an $r^2 = 0.9757$.

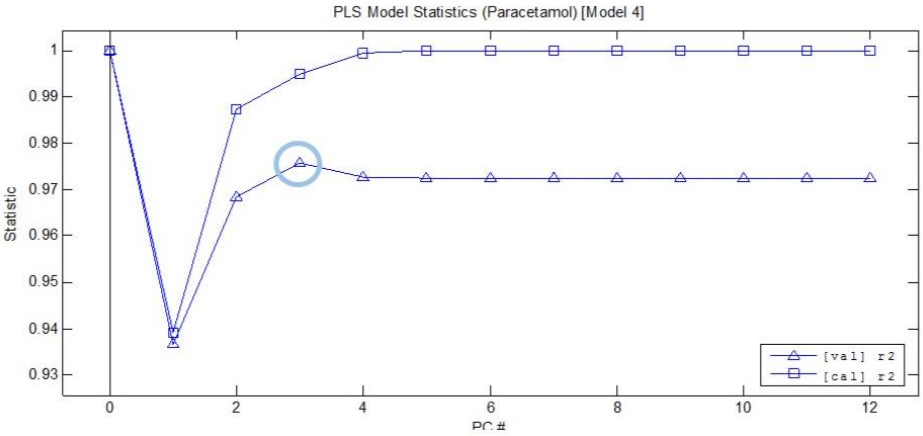

Figure 5.14 PLS model statistics for the validation method "take 1 tablet out". [PhAT probe] [Latentix™]

The possibility that predicting models could be more accurate if a more specific range of the spectrum was chosen, existed. Therefore, a small range based on the range where most paracetamol peaks are, was selected for the same predicting models. The selected spectrum is detached in Figure 5.15 and ranges from 1100 to 1700 cm^{-1}.

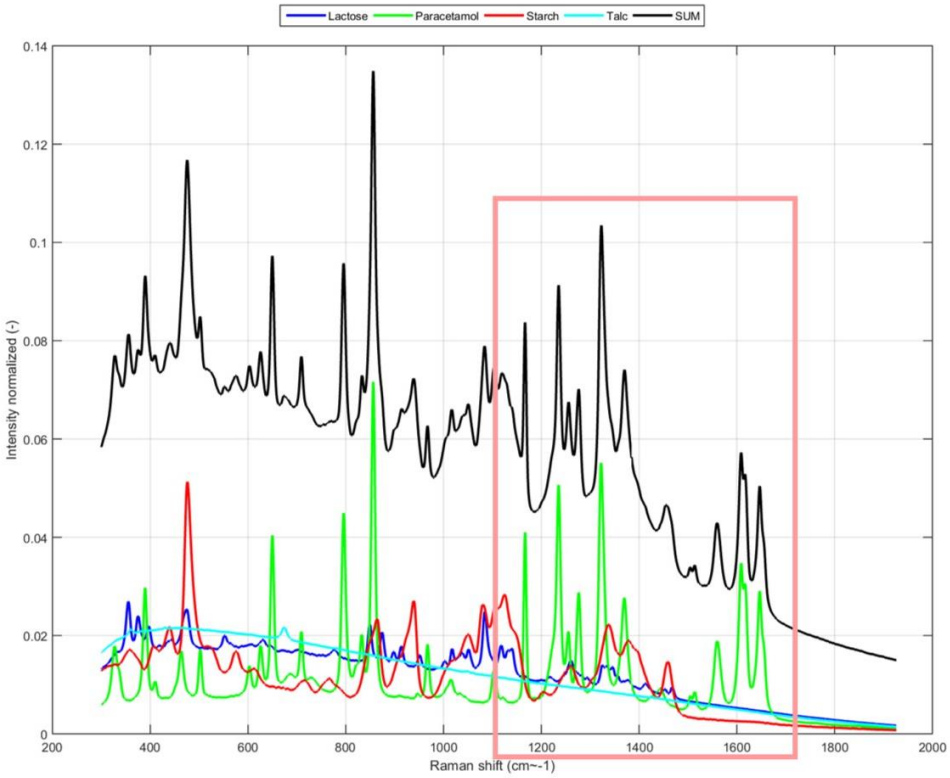

Figure 5.15 Spectrums of Paracetamol (green), Lactose (blue), Starch (red), Talk (light blue) and the SUM of all spectrums (black). Selected range selected for further analysis is detached with a colored square.

For this small range, the same procedures regarding preprocessing and PCA were performed and a PLS model was developed. For the validation method "*take 1 blend out*" Figure 5.16 shows that 1 PC is the ideal number of PC for this validation method, with an $r^2 = 0.9447$. This value is lower than the r^2 of the big range, for 3 PCs which means that using the entire range is a better choice.

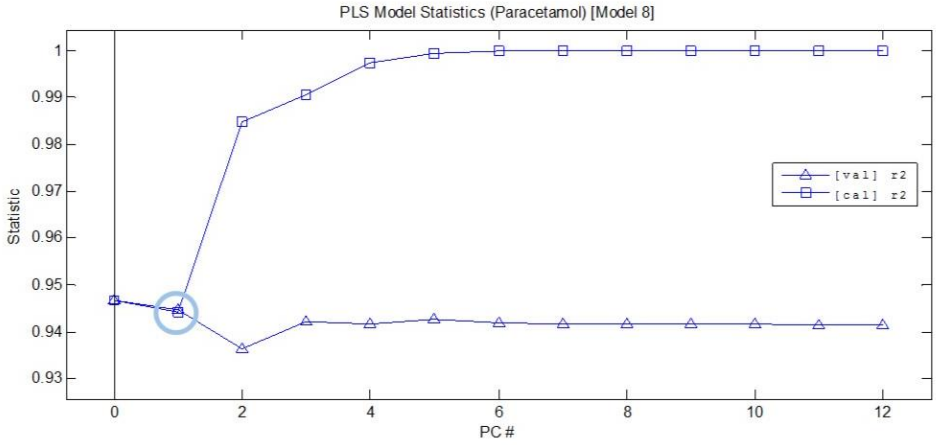

Figure 5.16 PLS model statistics for the validation method "take 1 blend out". [PhAT probe – small range] [Latentix™]

Figure 5.17 shows that 1 PC is the ideal number of PC for the validation method "*take 1 tablet out*", with an $r^2 = 0.9421$. This value is again lower than the r^2 of the big range, for 3 PCs which emphasizes that using the entire range is a better choice.

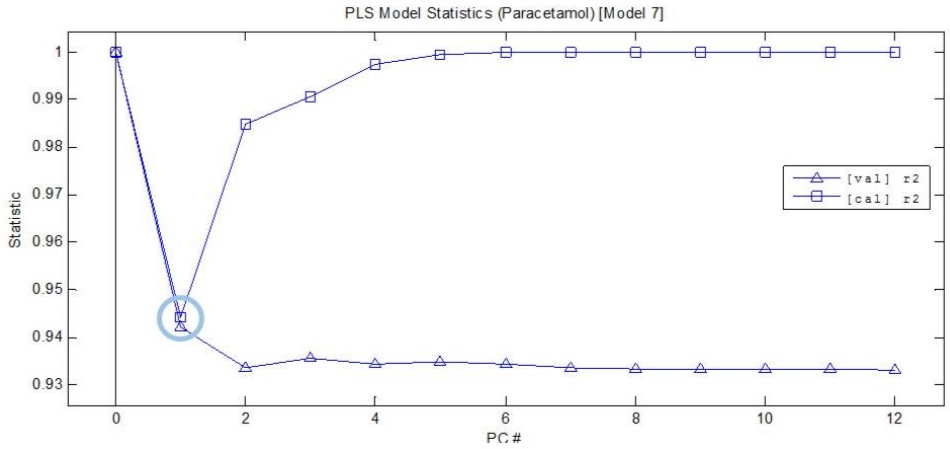

Figure 5.17 PLS model statistics for the validation method "take 1 tablet out". [PhAT probe – small range] [Latentix™]

5.3 MKII probe

The detection of different blends through white blister, using MKII probe was performed. Resulting spectra from these measurements as well as the effect of the different preprocessing methods can be found in Appendix II.

As observable in Figure 5.18, for the validation method "*take 1 blend out*" 5 PCs is the ideal number of PCs for the measurements with MKII probe, with an $r^2 = 0.8421$. This is not a very high r^2 value which indicates that this predicting model is not as robust as the previous one.

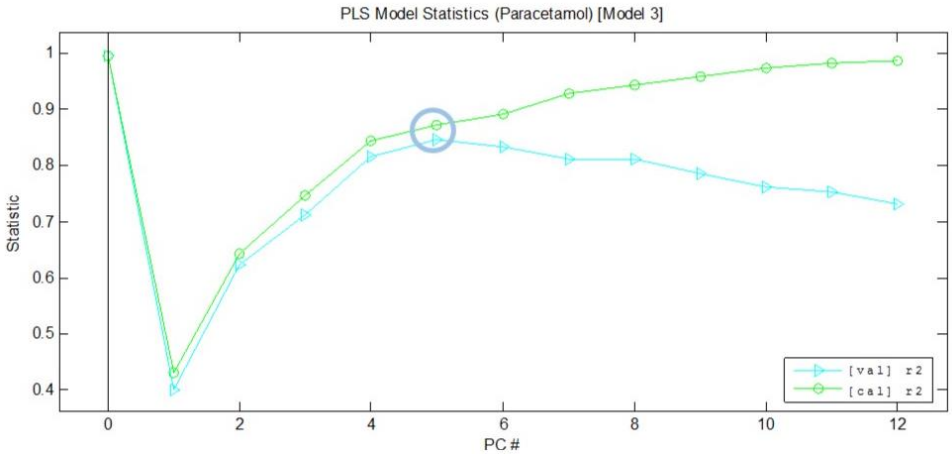

Figure 5.18 PLS model statistics for the validation method "take 1 blend out". [MKII probe] [Latentix™]

For the validation method "*take 1 tablet out*" 5 PCs is also the ideal number of PCs for describing the system, with an $r^2 = 0.8791$ as Figure 5.19 shows.

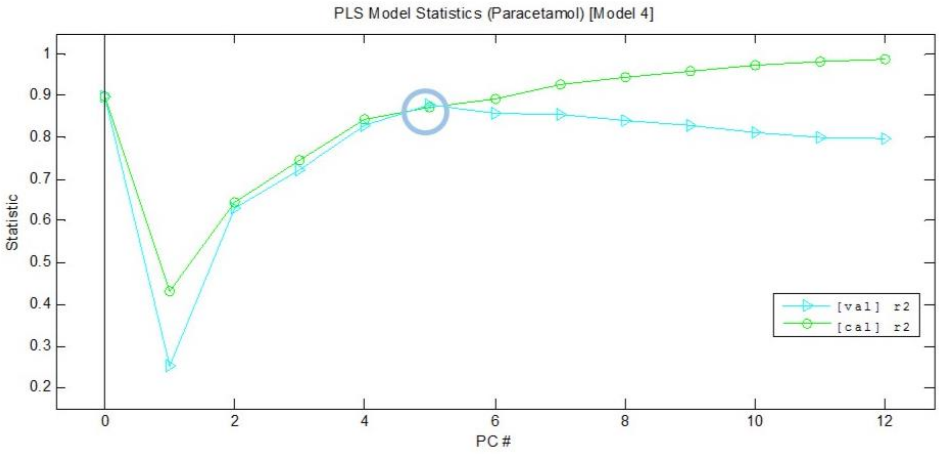

Figure 5.19 PLS model statistics for the validation method "take 1 tablet out". [MKII probe] [Latentix™]

For the small range, the same procedures regarding preprocessing and PCA were performed and a PLS model was developed, just like with PhAT probe. For the validation method "*take 1 blend out*" Figure 5.20 shows that 1 PC is the ideal number of PC for this validation method, with an $r^2 = 0.5108$. This value is low compared to the value obtained previously with PhAT probe measurements, which means that the model is not so robust.

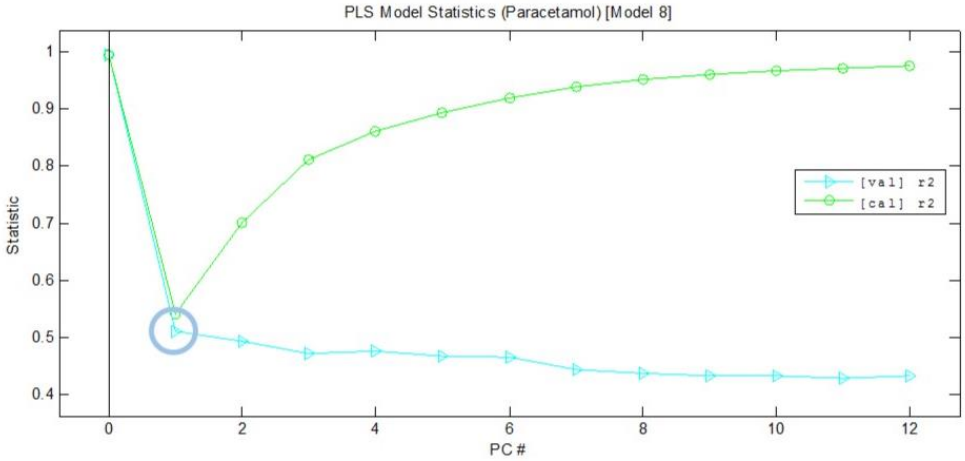

Figure 5.20 PLS model statistics for the validation method "take 1 blend out". [MKII probe – small range] [Latentix™]

Figure 5.21 shows that 1 PC is the ideal number of PC for the validation method "*take 1 tablet out*" (for the small range) with an $r^2 = 0.4961$, a low value.

The r^2 values obtained using the small range of the spectrums obtained with MKII probe are both lower than the r^2 values for the big range which also means that using the entire spectrum is better for the spectrum analysis and developing predicting models.

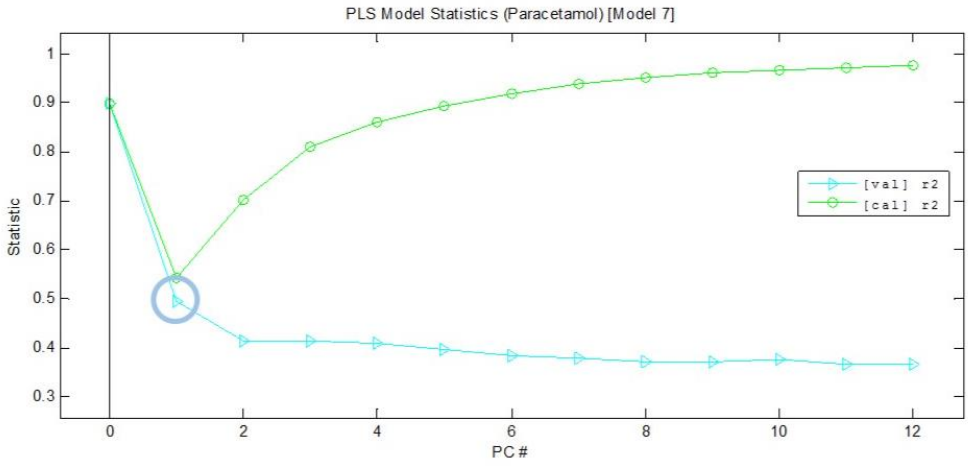

Figure 5.21 PLS model statistics for the validation method "take 1 tablet out". [MKII probe – small range] [Latentix™]

5.4 Green laser

The detection of different blends through white blister using a green laser was performed. Resulting spectra from these measurements as well as the effect of the different preprocessing methods can be found in the Appendix III.

As observable in Figure 5.22, for the validation method "*take 1 blend out*" 4 PCs is the ideal number of PCs for the measurements with the green laser, with an $r^2 = 0.4643$. This value is very low which means that this laser is no so good in detecting different concentration of paracetamol in tablets through white blisters. The prediction model is not robust enough to be trustful.

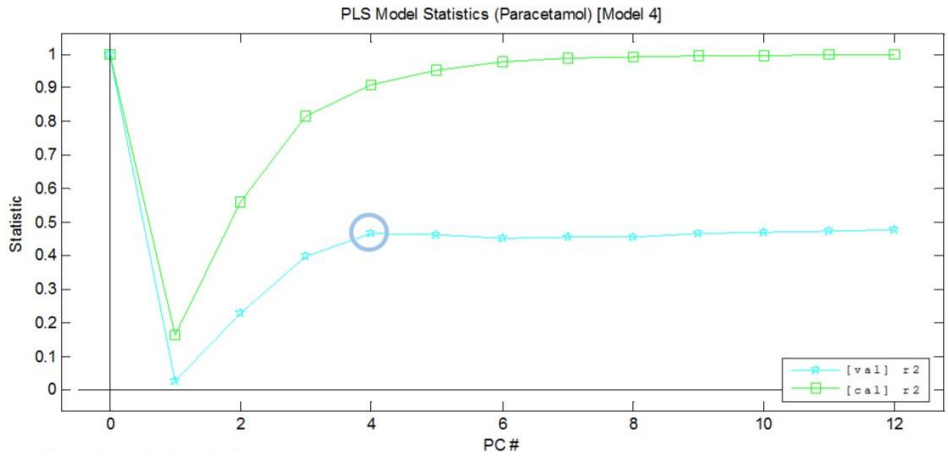

Figure 5.22 PLS model statistics for the validation method "take 1 blend out". [green laser] [Latentix™]

For the validation method "*take 1 tablet out*" 5 PCs is the ideal number of PCs for describing the system, with an $r^2 = 0.5619$ as Figure 5.23 shows.

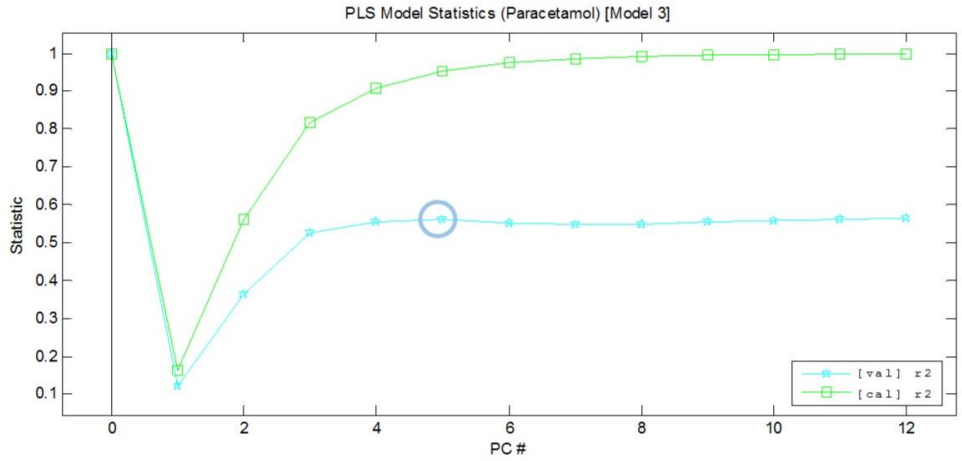

Figure 5.23 PLS model statistics for the validation method "take 1 tablet out". [green laser] [Latentix™]

For the small range, the same procedures regarding preprocessing and PCA were performed and a PLS model was developed. For the validation method "*take 1 blend out*" Figure 5.24 shows that 2 PCs is the ideal number of PC for this validation method, with an r^2 = 0,052. This value is unacceptable for the purpose.

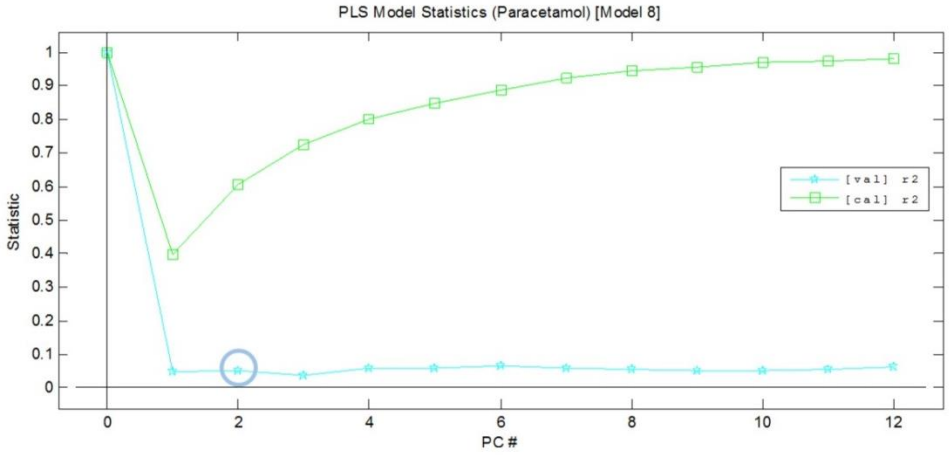

Figure 5.24 PLS model statistics for the validation method "take 1 blend out". [green laser – small range] [Latentix™]

Figure 5.25 shows that 1 PC is the ideal number of PC for the validation method "*take 1 tablet out*" (for the small range) with an r^2 = 0.179. This value is also unacceptable for the purpose of this investigation.

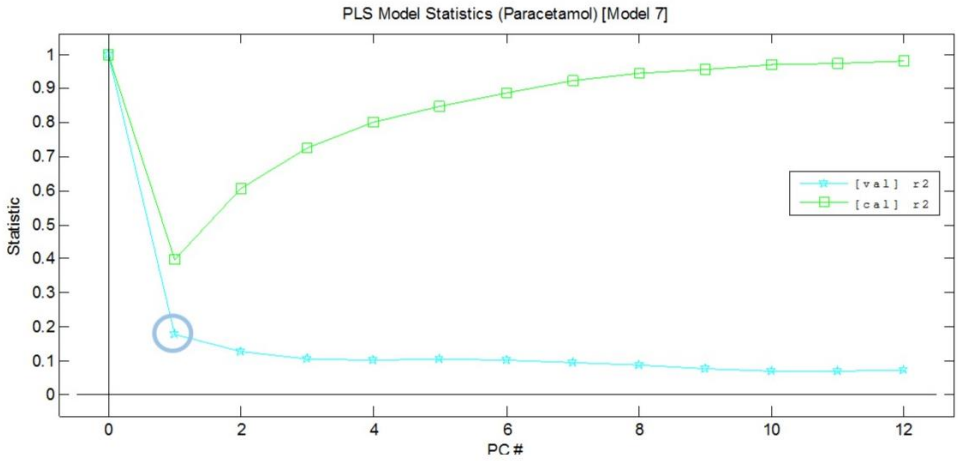

Figure 5.25 PLS model statistics for the validation method "take 1 tablet out". [green laser – small range] [Latentix™]

Chapter 6 - Conclusion

In this chapter, the main results presented in Chapter 5 are interrelated with the fundamental purpose of this investigation: detection of counterfeit medicines.

One of the most complex and challenging problems faced as a result of the globalization of health-care delivery is securing the integrity and safety of the global medicines supply chain (T. K. Mackey et al., 2015). Counterfeit medicines are widespread in all countries around the globe and represent a major public health concern, often resulting in treatment failure, serious deterioration of the state of health, or even death (Baratta et al., 2012). It is low- and middle-income countries and those in areas of conflict, or civil unrest, with very weak or non-existent health systems that bear the greatest burden of SSFFC medical products because the cost of legitimate drugs is beyond the reach of much of the population and legal controls are often weak (World Health Organization, 2010).

Since technology may improve the detection of low-quality drugs, it would be important that these methods will also be available in developing countries, where counterfeit products are the most common (Kelesidis & Falagas, 2015). Lack of inexpensive field screening technologies is one of the major impediments for their elimination/eradication and further scientific research and development is called for to design user-friendly, low-cost and robust portable (hand-held) devices and techniques for detecting and identifying counterfeit medicines in real settings (Karunamoorthi, 2014). The question remains as to how to use analytical methods in parts of the world with limited laboratory capacity and trained chemists because the most sophisticated analytic technologies were not designed for the field (IoM, 2013). Technologies used in the field for the detection of counterfeit medicines should ideally be portable, relatively easy to use, inexpensive to buy and maintain and resistant in order to be used in developing countries with little financial capacity and where the detection process must be fast and there is little specialized personnel. They also must provide reliable and useful data.

Raman spectroscopy is an established laser-based technology for the quality assurance of pharmaceutical products (Brauchle & Schenke-Layland, 2013). The intensity of Raman scattered light can be used to measure how much of a material is in a sample (quantitative analysis). The shape of a Raman spectrum can be used to determine the types of molecular vibrations in a sample. This vibrational information can be used to identify materials in a sample (for qualitative analysis) (Kaiser Optical Systems, 2004). The analytical versatility of Raman molecular spectroscopic techniques allows for investigating a wide diversity of transparent, translucent, opaque, and colored samples including solids, semi-solids, suspensions, and solutions (Paudel et al., 2015). Advantages of Raman spectroscopy include: high chemical specificity, the ability to quantify multiple constituents of a solid dose form, the high speed of analysis, the absence of sample preparation, the absence of solvents and/or consumables and the non-destructive nature of analysis compared with the traditional analytical techniques (Griffen et al., 2016).

Since the aim of this dissertation was to study de detection of medicines through its package, it was relevant to investigate if there were differences within the package material and how these differences could affect the spectrums of the tablets under analysis. The results revealed that in fact there are variations in package materials that influence the spectrums acquired. These variations can be due to thickness or density variations. Nevertheless, these variations can be easily overcome using preprocessing methods. In this case, SNV, SG and Mean Center were applied in this exact order, to minimize fluctuations. These simple treatments were, therefore, applied to all spectrums in this dissertation. The utilization of simple preprocessing methods is a very positive aspect for the treatment of data from measurements through packages because it simplifies its applicability in places without specialized personnel. Spectra from all measurements in this dissertation were analyzed using *Latentix™ software*. *Latentix™* was also developed with the intention to make low cost professional *software* for multivariate analysis so that Chemometrics and multivariate models could be available to everybody in all technological and scientific disciplines.

Aluminum blisters were also investigated to find some signal through this material. However, the results show that it is not possible to detect medicines through aluminum, using Raman spectroscopy (as expected). This is a disadvantage for the use of Raman spectroscopy for detecting counterfeits in a non-invasive way, however it can be overcome if the tablet is removed from the blister and analyzed directly.

For experiencing the detection of different concentrations of paracetamol through blister packages, calibration tablets were made for the purpose. Furthermore, in this investigation, three different probes were used for the measurements: PhAT probe, MKII probe attached to Raman Workstation microscope, and a green laser, to compare results. Results of these measurements are summarized in Table 6.1. From this table, some conclusions can be drawn:

● The best predicting models were accomplished using PhAT probe's measurements, where 3 PCs is the ideal number of PCs for both validation methods ("*leave 1 tablet out*" and "*leave 1 blend out*"), with an $r^2 \approx 0.98$ using the entire range.

● Since r^2 values related to the small range are lower than the r^2 values related to the big range, using the entire range of spectrums (big range) is a better option compared to using a smaller range because it gives rise to better predicting models.

● The green laser, representing handheld devices (cheaper and less accurate), is not stable/reproducible enough to guarantee a successful analysis of medicines through packages, when compared to the other techniques that are, however, more expensive.

Table 6.1 Comparison of r^2 values and corresponding number of PC for predicting models of both validation methods using measurements from PhAT probe, MKII probe and green laser

	Leave 1 tablet out				Leave 1 blend out			
	Big Range		**Small Range**		**Big Range**		**Small Range**	
	r^2	PC	r^2	PC	r^2	PC	r^2	PC
PhAT probe	0.9757	3	0.9421	1	0.9764	3	0.9447	1
MKII probe	0.8791	5	0.4961	1	0.8421	5	0.5108	1
Green laser	0.5619	5	0.1790	1	0.4643	4	0.052	2

Taking into account the three most important features for a measuring device to select for this purpose (which are robustness, cost and sampling), the best option can be analyzed in Table 6.2. In terms of robustness and versatility, the PhAT probe proved to be the best one although the MKII probe also presented high robustness values. In terms of cost, it is known that the PhAT probe and MKII probe are considerably more expensive than the green laser. Finally, considering sampling statistics, the PhAT probe is the best one because of its WAI scheme that enables larger samples compared to the MKII probe, for which it was necessary to perform three measurements of different spots for each tablet. In respect to the green laser, the sampling area is not really known. Hereupon, despite having a higher cost compared to the green laser, the best option is the PhAT probe.

Table 6.2 Comparison between three different probes considering robustness, cost and sampling.

	Robustness	Cost	Sampling
PhAT			
MK2			
Green			

Therefore, it is possible to detect different concentrations of an active ingredient of tablets through a white blister package using Raman spectroscopy, namely the PhAT probe. This equipment can be put in place analysis enabling faster detection of counterfeit medicines, in a non-invasive and non-destructive way that requires no sample preparation and does not need highly specialized personnel for its use. It provides reliable and useful data and it is portable. This is a promising technique for detecting counterfeit medicines and it is relatively low cost.

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Attachments

Appendix I

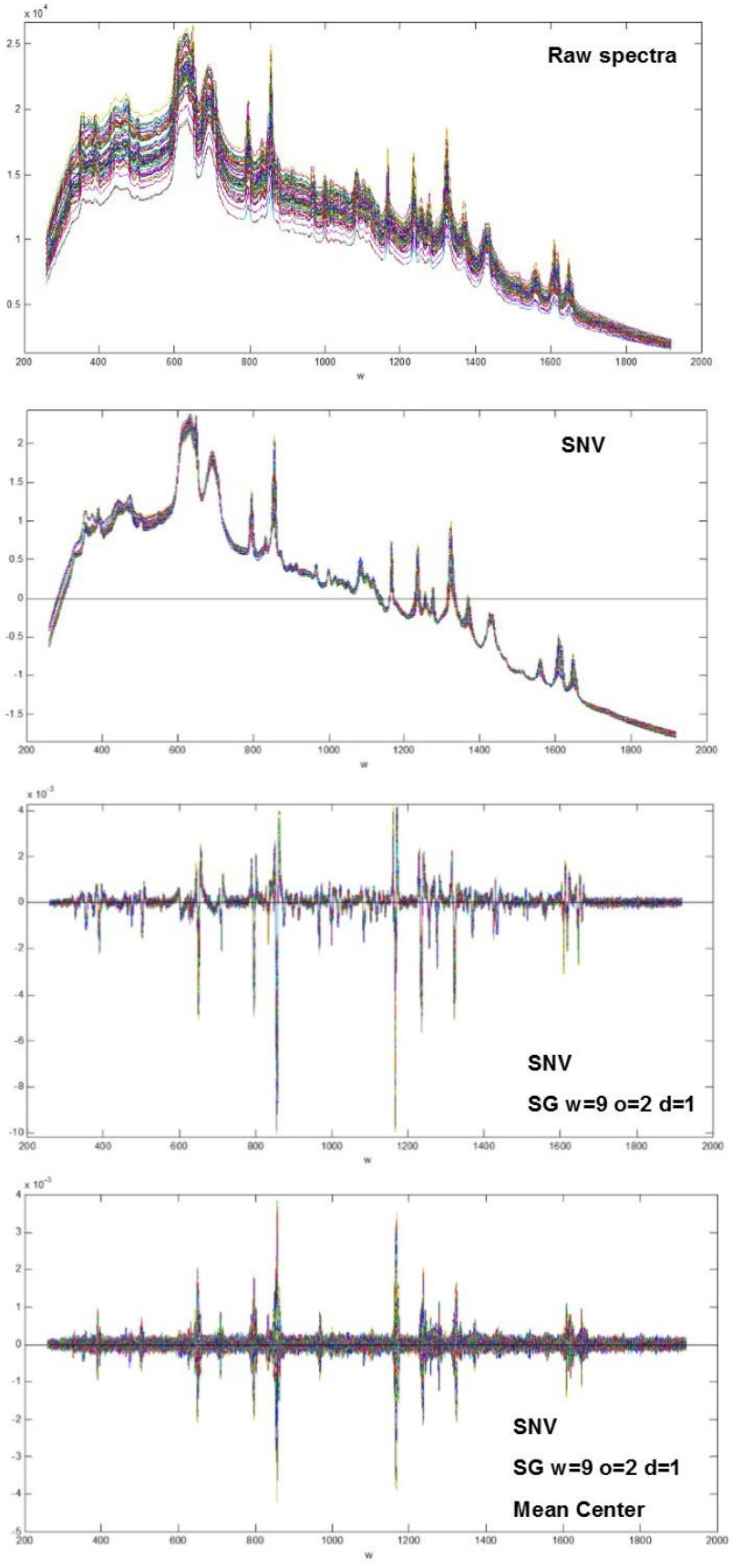

Figure I.1 The effect of preprocessing of Raman spectra of tablets (with different concentrations of paracetamol) measured through white blister packaging using PhAT probe from Kaiser Optical Systems [Latentix]

Appendix II

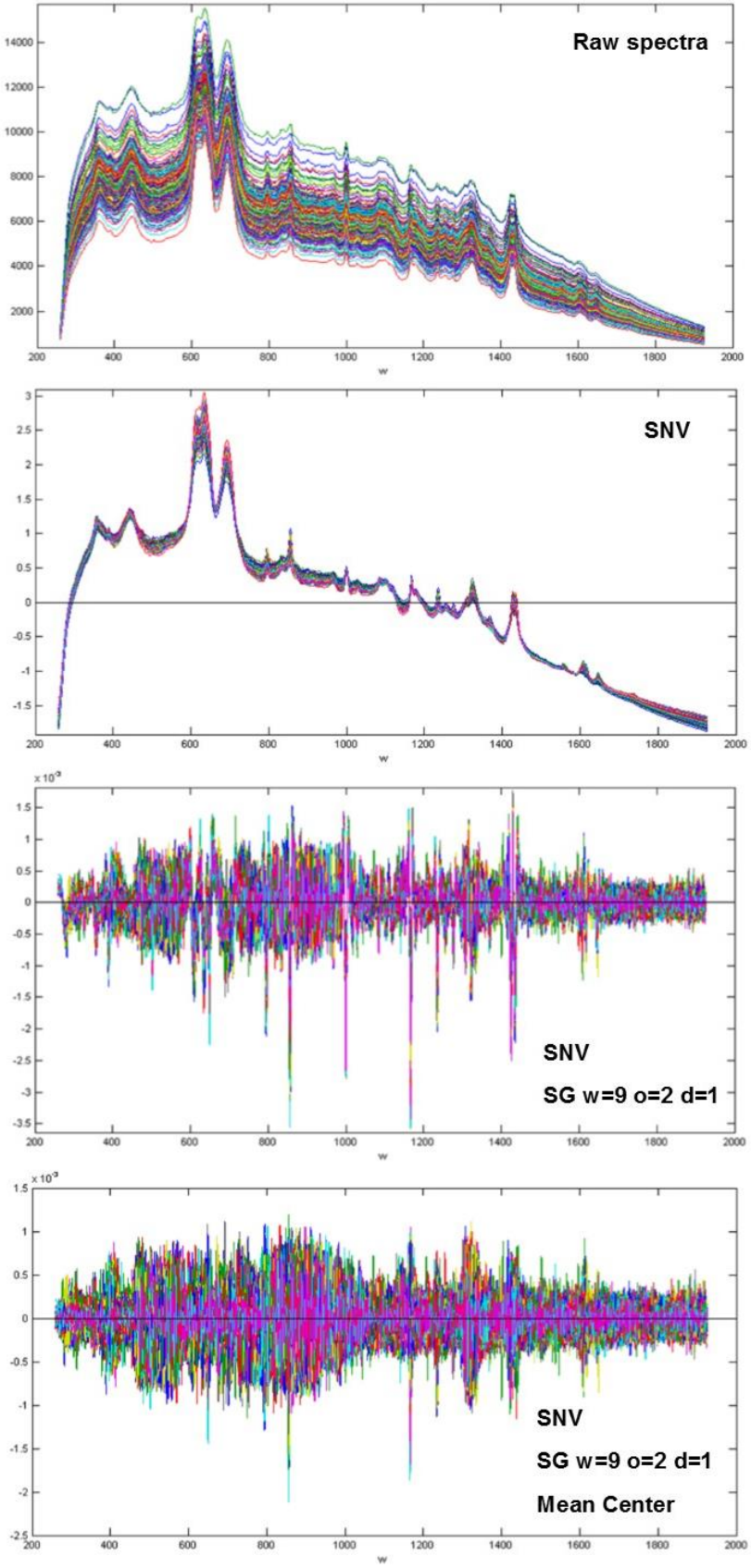

Figure II.1 The effect of preprocessing of Raman spectra of tablets (with different concentrations of paracetamol) measured through white blister packaging using MKII probe from Kaiser Optical Systems [Latentix]

Appendix III

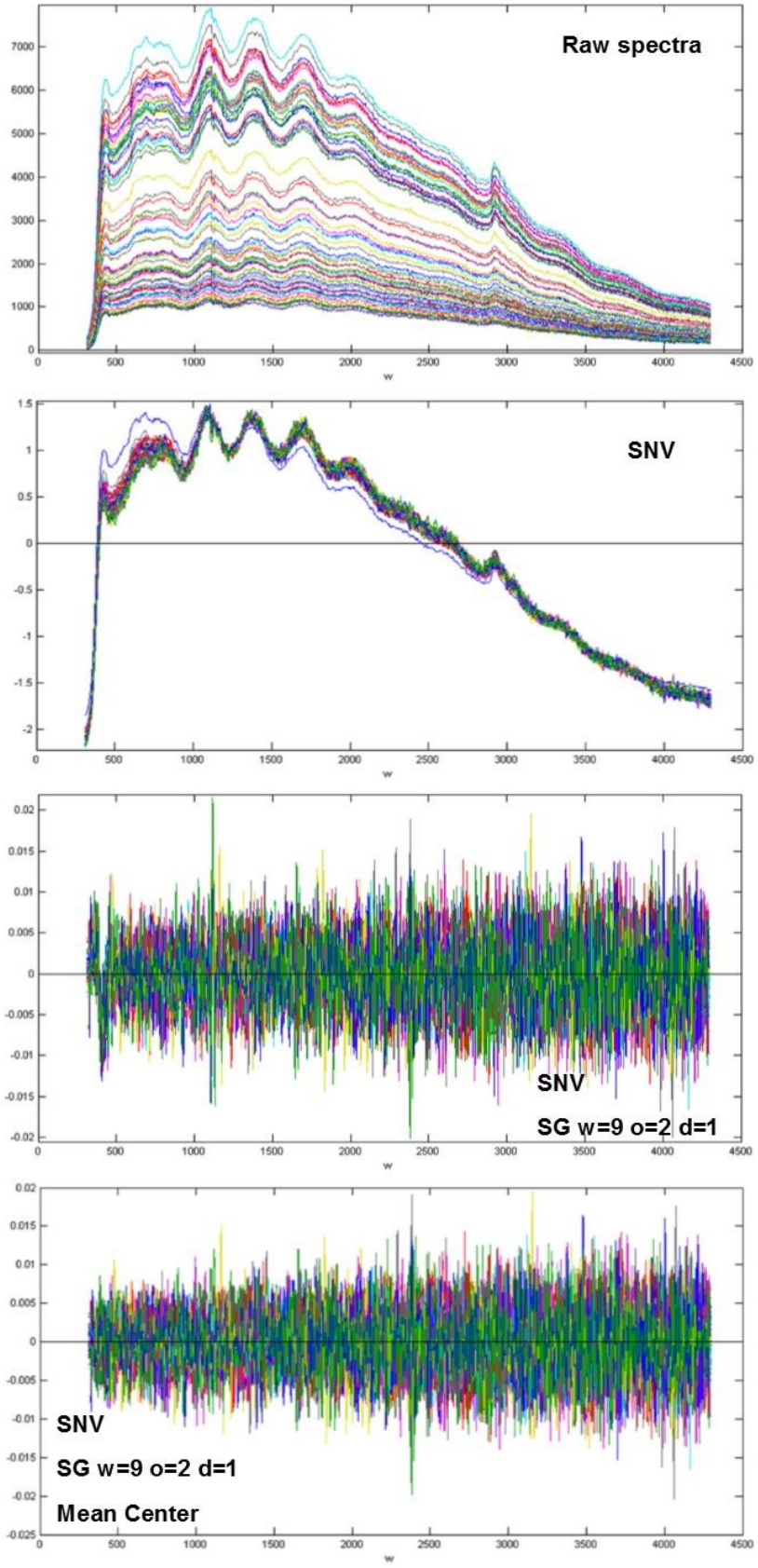

Figure III.1 The effect of preprocessing of Raman spectra of tablets (with different concentrations of paracetamol) measured through white blister packaging using the green laser [Latentix]